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| (60) Parent Application or Grant AMGEN INC. [/]; O. CITRON, Martin [/]; O. VASSAR, Robert, James [/]; O. BENNETT, Brian, Drake [/]; O. ODRE, Steven, M. ; () | | | |

(54) Title: BETA SECRETASE GENES AND POLYPEPTIDES

(54) Titre: GENES ET POLYPEPTIDES BETA SECRETASES

(57) Abstract

Disclosed are novel genes encoding beta secretase polypeptides. Also disclosed are methods of making and using the polypeptides.

(57) Abrégé

L'invention concerne de nouveaux gènes codant pour des polypeptides bêta sécrétase, ainsi que des méthodes de fabrication et d'utilisation des polypeptides.

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Description

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- 1 -

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BETA SECRETASE GENES AND POLYPEPTIDES

15 5 Field of the Invention

The present invention relates to novel beta secretase polypeptides and nucleic acid molecules encoding the polypeptides. The invention also relates to vectors, host cells, antibodies and methods for producing beta secretase polypeptides.

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25 Background of the Invention

Patients with Alzheimer's disease initially show short term memory loss. As the disease progresses, the patients become completely demented. The brains of Alzheimer's patients contain numerous amyloid plaques and neurofibrillary tangles (highly insoluble protein aggregates). Not surprisingly, such plaques and tangles are found primarily in those regions of the brain involved with memory and cognition.

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35 The amyloid plaques are associated with dystrophic dendrites and axons, as well as activated microglia and reactive astrocytes. The major component of the plaques is a peptide referred to as A-beta (Selkoe, 25 *Science*, 275:630-631 [1997]).

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A-beta is ultimately generated by endoprotease cleavages of the polypeptide beta-amyloid precursor protein (beta-APP). Beta-APP is constitutively expressed in most cells of the body, but 30 production of A-beta appears to be highest in brain cells. A-beta is typically secreted by the cells that produce it into the extracellular matrix where it makes its way into various bodily fluids. A schematic diagram of beta-APP processing is set forth in Figure 45 35 12.

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10 There are three major isoforms of beta-APP polypeptide, and they are referred to as beta-APP695, beta-APP751 and beta-APP770. The number on each isoform refers to the number of amino acids it
5 contains.

15 Beta-APP is synthesized as a membrane protein and spans the Golgi membrane. Normal non-pathogenic processing of beta-APP is believed to occur via a putative enzyme referred to as "alpha-secretase".

20 10 Alpha-secretase cleaves between amino acids 687 and 688 of beta-APP770 (or between amino acids 612 and 613 of beta-APP695), thereby generating a 687 amino acid soluble form of beta-APP770 (or a 612 amino acid soluble form of beta-APP695) referred to as "alpha-

25 15 APPs". The remaining membrane-bound portion of beta-APP (amino acids 688-770 of the beta-APP770 isoform) is cleaved by the enzyme gamma-secretase. Gamma secretase cleaves between amino acids 711 and 712, and between amino acids 713 and 714, to generate the fragments 688-

30 20 711, 688-713, and 712-770 and 714-770. The first two fragments are referred to as "p3" and are released from the membrane. Gamma secretase is also active on the two other isoforms of beta-APP.

35 In both non-diseased people and Alzheimer's 25 disease patients, beta-APP is also processed via an alternative mechanism. Here, a heretofore

40 30 uncharacterized enzyme referred to as "beta-secretase" cleaves full length beta-APP between amino acids 671 and 672 of beta-APP770, thereby generating a soluble fragment of amino acids 1-671, referred to as "beta-

45 35 APPs" and a membrane bound form of amino acids 672-770. The membrane bound form is then acted on by gamma secretase which, as mentioned above, cleaves between amino acids 711 and 712, and between amino acids 713 and 714 to produce soluble fragments of amino acids

50 672-711 and 672-713; these fragments are referred to as

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- 3 -

10 "A-beta 40", and "A-beta 42", respectively. In
addition, gamma secretase activity generates membrane
bound fragments of amino acids 712-770 and 714-770. A-
beta 40 and A-beta 42 are secreted from the cells and
15 accumulate in the brains of Alzheimer's patients to
form the characteristic brain tissue plaques.

Recent evidence suggests that beta secretase
can also cleave beta-APP between amino acids 683 and
684 of the 770 isoform (Gouras et al., *J. Neurochem.*,
10 71:1920-1925 [1998]) thereby ultimately generating a
20 second form of A-beta spanning amino acids 684-711 and
684-713. The mechanisms that control this alternate
cleavage are not known.

25 A few naturally occurring mutations of beta-
APP have been identified. These include the so called
"Swedish mutation" in which amino acids 670 and 671 of
the 770 isoform are mutated, and the London mutation in
30 which amino acid 717 of the 770 isoform is mutated.
The Swedish mutation, which causes familial Alzheimer's
disease, has been found to result in increased A-beta
20 production *in vivo* (Citron et al., *Nature*, 360:672-674
[1992]; see also Citron et al., *Neuron*, 14:661-670
[1995]).

35 US Patent No. 5,744,346 purportedly describes
25 a beta secretase molecule. No nucleic acid sequence or
amino acid sequence for this molecule is presented.

40 European patent application EP 0 855 444 A2
published 29 July 1998 describes an aspartic proteinase
that purportedly plays a role in Alzheimer's disease.
30 This aspartic proteinase has sequence similarity at
both the nucleic acid and amino acid levels to the
45 beta-secretase of the present invention.

European patent application EP 0 848 062 A2,
50 published 17 June 1998, describes an aspartic
35 proteinase termed "ASP1" which has approximately 47
percent identity at the amino acid level to the prepro

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- 4 -

10 form of the beta-secretase of the present invention as determined by a computer comparison using the GAP alignment program.

15 Accordingly, it is an object of the invention
5 to identify nucleic acid molecules encoding beta secretase polypeptides. Such molecules have use as probes for diagnosis of Alzheimer's disease, and for identification of compounds that modulate the activity of beta secretase.

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Summary of the Invention

25 The invention provides an isolated nucleic acid molecule selected from the group consisting of:

15 a) the nucleic acid molecule as set forth in any of SEQ ID NOS: 1, 2, and 3;

b) a nucleic acid molecule encoding the polypeptide of any of SEQ ID NOS: 4, 5, and 6;

30 c) biologically active fragments of SEQ ID NO:4;

d) an allelic variant or splice variant of any of (a) or (b);

35 e) a nucleic acid molecule of the DNA vector insert in ATCC Deposit No. 207158;

25 f) a nucleic acid molecule of the DNA vector insert in ATCC Deposit No. 207159;

40 g) a nucleic acid molecule encoding a polypeptide having one to fifty conservative amino acid substitutions as compared with the polypeptide of SEQ

30 ID NO:4, wherein the polypeptide encoded by said nucleic acid molecule is biologically active; and

45 h) a nucleic acid molecule that is the complement of any of (a)-(g) above.

In other embodiments, the invention provides
35 expression vectors, host cells, and methods of preparing recombinant beta secretase polypeptide.

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10 The invention also provides an isolated polypeptide selected from the group consisting of:

15 a) the polypeptide of any of SEQ ID NOS: 4, 5, and 6;

5 b) a biologically active fragment of any of SEQ ID NOS. 4, 5, 6;

15 c) a biologically active polypeptide having one to fifty conservative amino acid changes as compared with the polypeptide of SEQ ID NO:4;

20 d) the polypeptide encoded by the DNA vector insert of ATCC Deposit Nos. 207158 and 207159; and

25 e) a polypeptide that is an allelic variant or splice variant of (a).

25 The invention further provides an isolated

15 beta secretase polypeptide fragment of SEQ ID NO:4 selected from the group consisting of: amino acids 46-501; amino acids 46-460; amino acids 45-460; amino acids 1-460; amino acids 93-292; amino acids 93-293; amino acids 91-295; amino acids 90-295; amino acids 90-

30 300; amino acids 280-310; amino acids 62-420; amino acids 1-420; amino acids 62-460; amino acids 90-460; amino acids 62-501; amino acids 62-460; amino acids 93-

35 293; amino acids 90-293; amino acids 90-300; amino acids 62-420; amino acids 62-501; amino acids 1-420;

40 25 amino acids 46-420; amino acids 62-420; amino acids 73-420; amino acids 83-420; amino acids 90-420; amino acids 62-417; amino acids 73-417; amino acids 83-417;

45 amino acids 90-417; amino acids 62-410; amino acids 73-410; amino acids 83-410; amino acids 90-4104; amino acids 62-402; amino acids 73-402; amino acids 83-402;

50 and amino acids 90-40.

45 The invention further provides an antibody or fragment thereof specifically binding to beta secretase polypeptide.

55 35 The invention yet further provides a fusion polypeptide comprising full length or truncated beta-

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- 6 -

10 secretase polypeptide fused to a heterologous amino acid sequence such as the Fc portion of human IgG.

Description of the Figures

5 Figure 1A and 1B depict the cDNA sequence of human beta secretase (SEQ ID NO:1).

10 Figure 2A and 2B depict the cDNA sequence of mouse beta secretase (SEQ ID NO:2).

15 Figure 3A and 3B depict the cDNA sequence of rat beta secretase (SEQ ID NO:3).

20 Figure 4 depicts the putative amino acid sequence of human beta secretase polypeptide (SEQ ID NO:4).

25 Figure 5 depicts the putative amino acid sequence of mouse beta secretase polypeptide (SEQ ID NO:5).

30 Figure 6 depicts the putative amino acid sequence of rat beta secretase polypeptide (SEQ ID NO:6).

35 Figure 7 depicts a human multiple tissue Northern blot which was probed with a *PstI* DNA fragment of human beta-secretase cDNA extending from nucleotide 318 to nucleotide 1090. Lane 1 is pancreas; Lane 2 is kidney; Lane 3 is skeletal muscle; Lane 4 is liver; Lane 5 is lung; Lane 6 is placenta; Lane 7 is brain; Lane 8 is heart; Lane 9 is peripheral blood leukocytes; Lane 10 is colon; Lane 11 is small intestine; Lane 12 is ovary; Lane 13 is spinal cord; Lane 14 is prostate; Lane 15 is thymus; Lane 16 is spleen. The lower panel shows a control hybridization using a DNA probe for

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- 7 -

actin. RNA size markers (in kb) are indicated on the
10 left.

Figure 8 depicts a Western blot of a SDS gel
5 containing protein from either human 293 cells
transfected with beta-secretase cDNA or human brain.
15 Lane 1 contains high molecular weight standards; lane 2
contains immunoprecipitate of cell culture medium from
human 293 cells transiently transfected with beta-
20 secretase cDNA using preimmune serum; lane 3 contains
immunoprecipitate of cell culture medium from human 293
cells transiently transfected with beta-secretase cDNA
using beta-secretase antibody; lane 4 contains
25 immunoprecipitate of homogenate from human Alzheimer's
disease brain tissue using preimmune serum; lane 5
contains immunoprecipitate of homogenate from human
Alzheimer's disease brain tissue using antibody against
beta-secretase; lane 6 contains immunoprecipitate of
30 homogenate from human control (non-Alzheimer's disease)
brain tissue using preimmune serum; lane 7 contains
immunoprecipitate of homogenate from human control
(non-Alzheimer's disease) brain tissue using antibody
against beta-secretase; lane 8 contains low molecular
35 weight standards.

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Figure 9A depicts a graph of ELISA assays to
detect A-beta 42. APP expressing cells were
40 transfected with either vector plasmid (negative
control), the beta-secretase vector (positive control),
30 or beta-secretase vector containing the D93A mutation.

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Figure 9B depicts graph of ELISA assays to
detect A-beta 40. APP expressing cells were
transfected with either vector plasmid (negative
50 control), the beta-secretase vector (positive control),
35 or beta-secretase vector containing the D93A mutation..

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- 8 -

10 Figure 10 depicts an SDS gel of purified
human beta-secretase prepared as an Fc fusion. The
beta-secretase-Fc fusion is indicated as "fusion" in
15 5 Lane 2; "Fc" refers to an Fc fragment only. Molecular
weight markers are indicated in Lane 1.

15

20 Figure 11 is a graph depicting the ability of
human beta-secretase-Fc fusion to cleave various APP
peptides. The X-axis is the amount of enzyme in the
reaction mixture and the Y-axis is the amount of
25 10 cleavage (denoted as pmol product formed) of each of
three substrate peptides. "Sw" refers to the APP
peptide with the Swedish mutation; "Wt" refers to wild
25 15 type APP peptide; and "MV" refers to a mutated APP
peptide.

30 Figure 12 is a schematic representation of
the processing steps of beta-APP to generate A-beta.
20 20 APP can undergo cleavage by alpha-secretase to form a
secreted alpha-APP soluble fragment ("alpha-APPS"), and
a membrane bound fragment of about 13 kDa. The
membrane bound fragment can then be cleaved by gamma-
35 35 secretase to release a fragment referred to as "p3".
25 40 Alternatively, APP can undergo beta-secretase cleavage
to release a soluble fragment referred to as "beta-
APPs" and a membrane bound fragment of about 12 kDa.
The membrane bound fragment can then be cleaved by
gamma-secretase to release A-beta.

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45 Detailed Description of the Invention

50 The section headings herein are for
organizational purposes only and are not to be
35 50 construed as limiting the subject matter described
therein.

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Definitions

15

The term "beta secretase nucleic acid molecule" refers to a nucleic acid molecule comprising or consisting essentially of a nucleotide sequence as set forth in any of SEQ ID NOS: 1, 2, and 3, comprising or consisting essentially of a nucleotide sequence encoding any of the polypeptides as set forth in SEQ ID NOS: 4, 5, and 6, comprising or consisting essentially of a nucleotide sequence of the DNA insert in ATCC deposit number 207158 and ATCC deposit number 207159 (both deposited 11 March 1999), or nucleic acid molecules related thereto. Related nucleic acid molecules comprise or consist essentially of nucleotide sequences that are at least 70 percent identical to the nucleotide sequence as shown in any of SEQ ID NOS: 1, 2, and 3, or comprise or consist essentially of nucleotide sequences encoding polypeptides that are at least 70 percent identical to any of the polypeptides as set forth in SEQ ID NOS: 4, 5, and 6. The nucleotide sequences may be at least 75 percent, or about 80 percent, or about 85 percent, or about 90 percent, or about 95 percent identical to any of the nucleotide sequences as shown in SEQ ID NOS: 1, 2, and 3, or the nucleotide sequences that encode polypeptides that are about 75 percent, or about 80 percent, or about 85 percent, or about 90 percent, or about 95 percent identical to any of the polypeptide sequences as set forth in SEQ ID NOS 4, 5, and 6. Related nucleic acid molecules also include fragments of the above beta secretase nucleic acid molecules which are at least about 10 contiguous nucleotides, or about 15, or about 20, or about 25, or about 50, or about 75, or about 100, or greater than about 100 contiguous nucleotides. Related nucleic acid molecules also include fragments of the above beta secretase nucleic acid molecules

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which encode polypeptides of at least about 25 amino acid residues, or about 50, or about 75, or about 100, or greater than about 100 amino acid residues. Related beta secretase nucleic acid molecules include those
10 molecules which comprise nucleotide sequences which hybridize under moderate or highly stringent conditions as defined herein with any of the above nucleic acid molecules. In preferred embodiments, the related nucleic acid molecules comprise sequences which
15 hybridize under moderate or highly stringent conditions with the sequence as shown in SEQ ID NO:1, or with a molecule encoding a polypeptide, which polypeptide comprises the sequence as shown in SEQ ID NO:4, or with a nucleic acid fragment as defined above, or with a
20 nucleic acid fragment encoding a polypeptide as defined above. It is also understood that related nucleic acid molecules include allelic or splice variants of any of the above nucleic acids, and include sequences which
25 are complementary to any of the above nucleotide
30 sequences.

The term "isolated nucleic acid molecule" refers to a nucleic acid molecule of the invention that is free from at least one contaminating nucleic acid molecule with which it is naturally associated, and
35 preferably substantially free from any other contaminating mammalian nucleic acid molecules which would interfere with its use in protein production or its therapeutic or diagnostic use.

The term "allelic variant" refers to one of several possible naturally occurring alternate forms of a gene occupying a given locus on a chromosome of an
40 organism.

The term "splice variant" refers to a nucleic acid molecule, usually RNA, which is generated
45 by alternative processing of intron sequences in an RNA transcript.

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- 11 -

10 The term "expression vector" refers to a
vector which is suitable for propagation in a host cell
and contains nucleic acid sequences which direct and/or
control the expression of inserted heterologous nucleic
5 acid sequences. Expression includes, but is not
limited to, processes such as transcription,
15 translation, and RNA splicing, if introns are present.

20 The term "high stringency conditions" refers
to those conditions that (1) employ low ionic strength
10 reagents and high temperature for washing, for example,
0.015 M NaCl/0.0015 M sodium citrate/0.1% NaDODSO4
(SDS) at 50°C, or (2) employ during hybridization a
25 denaturing agent such as formamide, for example, 50%
(vol/vol) formamide with 0.1% bovine serum
25 albumin/0.1%. Alternatively, Ficoll/0.1%
polyvinylpyrrolidone/50 mM sodium phosphate buffer at
pH 6.5 may be used with 750 mM NaCl, 75 mM sodium
30 citrate at 42°C. Another example is the use of 50%
formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium
35 citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium
pyrophosphate, 5 x Denhardt's solution, sonicated
salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran
sulfate at 42°C, with washes at 42°C in 0.2 x SSC and
0.1% SDS.

40 The term "moderate stringency conditions"
refers to conditions which generally include the use of
a washing solution and hybridization conditions (e.g.,
temperature, ionic strength, and percent SDS) less
45 stringent than described above. An example of
30 moderately stringent conditions are conditions such as
overnight incubation at 37°C in a solution comprising:
20% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium
citrate), 50 mM sodium phosphate (pH 7.6), 5 x
50 Denhardt's solution, 10% dextran sulfate, and 20 µl/ml

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- 12 -

denatured sheared salmon sperm DNA, followed by washing
the filters in 1 x SSC at about 37-50°C. The skilled
artisan will recognize how to adjust the temperature,
ionic strength etc. as necessary to accommodate factors
such as probe length and the like.

Where oligonucleotide probes are used to
screen cDNA or genomic libraries, one of the following
two high stringency solutions may be used. The first
of these is 6 X SSC with 0.05 percent sodium
pyrophosphate at a temperature of 35°C-62°C, depending
on the length of the oligonucleotide probe. For
example, 14 base pair probes are washed at 35-40°C, 17
base pair probes are washed at 45-50°C, 20 base pair
probes are washed at 52-57°C, and 23 base pair probes
are washed at 57-63°C. The temperature can be
increased 2-3°C where the background non-specific
binding appears high. A second high stringency
solution utilizes tetramethylammonium chloride (TMAC)
for washing oligonucleotide probes. One stringent
washing solution is 3 M TMAC, 50 mM Tris-HCl, pH 8.0,
and 0.2 percent SDS. The washing temperature using
this solution is a function of the length of the probe.
For example, a 17 base pair probe is washed at about
45-50°C.

The term "beta secretase polypeptide" refers
to a polypeptide comprising the amino acid sequence of
any of SEQ ID NOS: 4, 5, and 6, and related
polypeptides described herein. Related polypeptides
includes allelic variants, splice variants, fragments,
derivatives, substitution, deletion, and insertion
variants, fusion polypeptides, and orthologs. Beta
secretase polypeptides may be processed polypeptides,
i.e., not containing an endogenous or exogenous signal
or leader sequence as defined herein, and may or may

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- 13 -

not have an amino terminal methionine residue,
depending on the method by which they are prepared.

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The term "mature beta-secretase polypeptide" refers to a polypeptide of any of SEQ ID NOS: 4, 5 and 6 and related polypeptides described herein, in which the leader sequence and the propeptide have been removed. Mature human beta-secretase is amino acids 46-501 of SEQ ID NO:4. For human beta-secretase, the leader peptide is amino acids 1-21 of SEQ ID NO:4, and the propeptide is amino acids 22-45 of SEQ ID NO:4.

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The term "beta secretase polypeptide fragment" refers to a peptide or polypeptide that comprises less than the full length amino acid sequence of an beta secretase polypeptide as set forth in any of SEQ ID NOS: 4, 5, and 6. Such a fragment may arise, for example, from a truncation at the amino terminus, a truncation at the carboxy terminus, and/or an internal deletion of a residue(s) from the amino acid sequence. Naturally occurring beta secretase fragments may result from alternative RNA splicing, from *in vivo* processing such as removal of the leader peptide and propeptide, and/or from protease activity.

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The term "beta secretase polypeptide variants" refers to beta secretase polypeptides comprising amino acid sequences which contain one or more amino acid sequence substitutions, deletions, and/or additions as compared to the beta secretase polypeptide amino acid sequence set forth in any of SEQ ID NOS: 4, 5, and 6 or fragments thereof. Variants may be naturally occurring or artificially constructed. Such beta secretase polypeptide variants may be prepared from the corresponding nucleic acid molecules encoding said variants, which have a DNA sequence that varies accordingly from the DNA sequences for wild type beta secretase polypeptides as set forth in any of SEQ ID NOS: 4, 5, and 6.

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- 14 -

10 The term "beta secretase fusion polypeptide" refers to a fusion of beta secretase polypeptide, fragment, variant and/or derivative thereof, with a heterologous peptide or polypeptide.

15 5 The term "beta secretase polypeptide derivatives" refers to beta secretase polypeptides, variants, or fragments thereof, that have been chemically modified, as for example, by covalent attachment of one or more water soluble polymers,

20 10 N-linked or O-linked carbohydrates, sugars, phosphates, and/or other such molecules. The derivatives are modified in a manner that is different from naturally occurring beta secretase, either in the type and/or location of the molecules attached to the polypeptide

25 15 Derivatives further include the deletion of one or more chemical groups naturally attached to the beta secretase polypeptide.

30 30 The terms "biologically active beta secretase polypeptides", "biologically active beta secretase 20 polypeptide fragments", "biologically active beta secretase polypeptide variants", and "biologically active beta secretase polypeptide derivatives" refer to beta secretase polypeptides having at least one activity characteristic of a beta secretase

35 25 polypeptide, such as the ability to cleave the APP Swedish mutation peptide EVKMDAEL (SEQ ID NO:18) between the methionine and aspartic acid residues.

40 40 The term "isolated polypeptide" refers to a polypeptide of the invention that is free from at least 30 one contaminating polypeptide that is found in its natural environment, and preferably substantially free 45 from any other contaminating mammalian polypeptides which would interfere with its therapeutic or diagnostic use.

45 35 The term "ortholog" refers to a polypeptide 50 that corresponds to a polypeptide identified from a

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10 different species. For example, mouse and human beta secretase polypeptides are considered orthologs.

The terms "effective amount" and "therapeutically effective amount" refer to the amount 5 of a beta secretase polypeptide that is useful or necessary to support an observable level one or more biological activities of the beta secretase polypeptides as set forth above.

10 **Relatedness of Nucleic Acid Molecules and/or Polypeptides**

20 The term "identity", as known in the art, refers to a relationship between the sequences of two or more polypeptide molecules or two or more nucleic acid molecules, as determined by comparing the 25 sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or nucleic acid molecule sequences, as the case may be, as determined by the match between strings of nucleotide 30 or amino acid sequences. "Identity" measures the percent of identical matches between two or more sequences with gap alignments addressed by a particular mathematical model or computer programs (i.e., "algorithms").

35 The term "similarity" is a related concept, but in contrast to "identity", refers to a measure of similarity which includes both identical matches and conservative substitution matches. Since conservative 40 substitutions apply to polypeptides and not nucleic acid molecules, similarity only deals with polypeptide 45 sequence comparisons. If two polypeptide sequences have, for example, 10/20 identical amino acids, and the remainder are all non-conservative substitutions, then the percent identity and similarity would both be 50%. 50 If in the same example, there are 5 more positions where there are conservative substitutions, then the

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percent identity remains 50%, but the per cent
similarity would be 75% (15/20). Therefore, in cases
where there are conservative substitutions, the degree
of similarity between two polypeptide sequences will be
higher than the percent identity between those two
sequences.

The term "conservative amino acid substitution" refers to a substitution of a native amino acid residue with a nonnative residue such that
there is little or no effect on the polarity or charge of the amino acid residue at that position. For example, a conservative substitution results from the replacement of a non-polar residue in a polypeptide with any other non-polar residue. Furthermore, any native residue in the polypeptide may also be substituted with alanine, as has been previously described for "alanine scanning mutagenesis". General classes of amino acids useful for conservative amino acid substitutions are set forth in Table I.

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Table I

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Conservative Amino Acid Substitutions

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| | | |
|----|------------------|--|
| | Basic: | arginine lysine histidine |
| | Acidic: | glutamic acid aspartic acid |
| | Uncharged Polar: | glutamine asparagine serine threonine tyrosine |
| 20 | Non-Polar: | phenylalanine tryptophan cysteine glycine alanine valine proline methionine |
| 25 | | leucine norleucine isoleucine |

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Conservative modifications to the amino acid sequence (and the corresponding modifications to the encoding nucleotides) are expected to produce beta secretase having functional and chemical characteristics similar to those of naturally occurring beta secretase.

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Conservative amino acid substitutions also encompass non-naturally occurring amino acid residues

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- 18 -

which are typically incorporated by chemical peptide synthesis rather than by synthesis in biological systems. Such conservative amino acids include, for example, the "homolog" of each amino acid, where the 5 homolog is an amino acid with a methylene group (CH₂) inserted into the side chain at the beta position of that side chain.

Beta-secretase polypeptides of the present invention may have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 10 12, 13, 14, 15, 20, 25, 30, 35, 40, 45, 50, or up to 55 20 conservative amino acid changes as compared with the beta-secretase polypeptide of SEQ ID NO:4. Such molecules may also have chemical modifications as described herein for beta-secretase variants.

In contrast, non-conservative substitutions 25 of beta secretase may be generated by selecting substitutions that differ significantly in their effect 30 on maintaining (a) the structure of the molecular backbone in the area of the substitution, for example, 35 20 as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Non-conservative 40 substitutions may involve the exchange of a member of 45 one of the amino acid classes of Table I for a member 25 from another class. Such substituted residues may be introduced into regions of the human beta secretase molecule that are homologous with non-human beta secretase, or into the non-homologous regions of the 50 molecule.

Identity and similarity of related nucleic 45 acid molecules and polypeptides can be readily calculated by known methods, including but not limited to those described in *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed.. Academic Press, New York, 1993;

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- 19 -

10 Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence
5 Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48:1073 (1988).

15 Preferred methods to determine identity and/or similarity are designed to give the largest
20 match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not
25 limited to, the GCG program package, including GAP (Devereux, et al., Nucleic Acids Research 12:387 (1984); Genetics Computer Group, University of Wisconsin, Madison, WI), BLASTP, BLASTN, and FASTA (Atschul et al., J. Molec. Biol. 215:403-410 [1990]).
30 The BLAST X program is publicly available from the National Center for Biotechnology Information (NCBI) and other sources (BLAST Manual, Altschul] et al., NCB
35 NLM NIH Bethesda, MD 20894; Altschul et al., J. Mol. Biol. 215:403-410 [1990]). The well known Smith Waterman algorithm may also be used to determine
40 identity.

45 By way of example, using the computer algorithm GAP (Genetics Computer Group, University of Wisconsin, Madison, WI), two polypeptides for which the
50 30 percent sequence identity is to be determined are aligned for optimal matching of their respective amino acids (the "matched span", as determined by the algorithm). A gap opening penalty (which is calculated as 3 X the average diagonal; the "average diagonal" is
55 35 the average of the diagonal of the comparison matrix being used; the "diagonal" is the score or number

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10 assigned to each perfect amino acid match by the particular comparison matrix) and a gap extension penalty (which is usually 1/10 times the gap opening penalty), as well as a comparison matrix such as PAM
15 5 250 or BLOSUM 62 are used in conjunction with the algorithm. A standard comparison matrix (see Dayhoff et al., in: *Atlas of Protein Sequence and Structure*, vol. 5, supp. 3 [1978] for the PAM250 comparison matrix; see Henikoff et al., *Proc. Natl. Acad. Sci. USA*,
20 10 89:10915-10919 [1992] for the BLOSUM 62 comparison matrix) is also used by the algorithm.

25 Preferred parameters for polypeptide sequence comparison include the following:

15

- Algorithm: Needleman and Wunsch, *J. Mol. Biol.* 48:443-453 (1970)
- Comparison matrix: BLOSUM 62 from Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915-10919
30 20 (1992)
- Gap Penalty: 12
- Gap Length Penalty: 4
- Threshold of Similarity: 0

35 25 The GAP program is useful with the above parameters. The aforementioned parameters are the default parameters for polypeptide comparisons (along with no penalty for end gaps) using the GAP algorithm.

40 30

Preferred parameters for nucleic acid molecule sequence comparison include the following:

45 35

- Algorithm: Needleman and Wunsch, *J. Mol. Biol.* 48:443-453 (1970)
- Comparison matrix: matches = +10, mismatch = 0

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- 21 -

- 10 • Gap Penalty: 50
 • Gap Length Penalty: 3

15 The GAP program is also useful with the above
5 parameters. The aforementioned parameters are the
default parameters for nucleic acid molecule
comparisons.

20 Other exemplary algorithms, gap opening
penalties, gap extension penalties, comparison
10 matrices, thresholds of similarity, etc. may be used by
those of skill in the art, including those set forth in
the Program Manual, Wisconsin Package, Version 9,
September, 1997. The particular choices to be made
will depend on the specific comparison to be made, such
25 as DNA to DNA, protein to protein, protein to DNA; and
additionally, whether the comparison is between given
15 pairs of sequences (in which case GAP or BestFit are
generally preferred) or between one sequence and a
large database of sequences (in which case FASTA or
30 BLASTA are preferred).

Nucleic Acid Molecules

35 Recombinant DNA methods used herein are
generally those set forth in Sambrook et al. (*Molecular
25 Cloning: A Laboratory Manual*, Cold Spring Harbor
Laboratory Press, Cold Spring Harbor, NY [1989]) and/or
Ausubel et al., eds., (*Current Protocols in Molecular
40 Biology*, Green Publishers Inc. and Wiley and Sons, NY
[1994]).

45 30 The invention provides for nucleic acid
molecules as described herein and methods for obtaining
the molecules. A gene or cDNA encoding a beta
secretase polypeptide or fragment thereof may be
obtained by hybridization screening of a genomic or
35 cDNA library, or by PCR amplification. Probes or

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- 22 -

primers useful for screening a library by hybridization can be generated based on sequence information for other known genes or gene fragments from the same or a related family of genes, such as, for example, 10
5 conserved motifs. In addition, where a gene encoding beta secretase polypeptide has been identified from one species, all or a portion of that gene may be used as a probe to identify corresponding genes from other 15
species (orthologs) or related genes from the same 10 species (homologs). The probes or primers may be used 20 to screen cDNA libraries from various tissue sources believed to express the beta secretase gene. In addition, part or all of a nucleic acid molecule having 25 any of the sequences as set forth in SEQ ID NOS: 1, 2, 15 and 3 may be used to screen a genomic library to identify and isolate a gene encoding beta secretase. Typically, conditions of moderate or high stringency will be employed for screening to minimize the number 30 of false positives obtained from the screen.
35 20 Nucleic acid molecules encoding beta secretase polypeptides may also be identified by expression cloning which employs detection of positive clones based upon a property of the expressed protein. Typically, nucleic acid libraries are screened by 40 25 binding of an antibody or other binding partner (e.g., receptor or ligand) to cloned proteins which are expressed and displayed on the host cell surface. The antibody or binding partner is modified with a detectable label to identify those cells expressing the 30 desired clone.
45 Another means of preparing a nucleic acid molecule encoding a beta secretase polypeptide or fragment thereof is chemical synthesis using methods well known to the skilled artisan such as those 50 35 described by Engels et al. (*Angew. Chem. Int'l. Ed.*, 28:716-734 [1989]). These methods include, *inter alia*,

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- 23 -

the phosphotriester, phosphoramidite, and H-phosphonate methods for nucleic acid synthesis. A preferred method for such chemical synthesis is polymer-supported synthesis using standard phosphoramidite chemistry.

- 10 5 Typically, the DNA encoding the beta secretase polypeptide will be several hundred nucleotides in length. Nucleic acids larger than about 100 nucleotides can be synthesized as several fragments using these methods. The fragments can then be ligated
- 15 10 together to form the full length beta secretase polypeptide. Usually, the DNA fragment encoding the amino terminus of the polypeptide will have an ATG, which encodes a methionine residue. This methionine may or may not be present on the mature form of the
- 20 15 beta secretase polypeptide, depending on whether the polypeptide produced in the host cell is designed to be secreted from that cell.

25 30 In some cases, it may be desirable to prepare nucleic acid molecules encoding beta secretase

- 35 20 polypeptide variants. Nucleic acid molecules encoding variants may be produced using site directed mutagenesis, PCR amplification, or other appropriate methods, where the primer(s) have the desired point mutations (see Sambrook et al., *supra*, and Ausubel et 40 25 al., *supra*, for descriptions of mutagenesis techniques). Chemical synthesis using methods described by Engels et al., *supra*, may also be used to prepare such variants. Other methods known to the skilled artisan may be used as well.

- 45 30 In one embodiment, nucleic acid molecule variants contain codons which have been altered for optimal expression of a beta secretase polypeptide in a given host cell. Particular codon alterations will depend upon the beta secretase polypeptide(s) and host 50 35 cell(s) selected for expression. Such "codon optimization" can be carried out by a variety of

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- 24 -

10 methods, for example, by selecting codons which are
preferred for use in highly expressed genes in a given
host cell. Computer algorithms which incorporate codon
frequency tables such as "Ecohigh.Cod" for codon
5 preference of highly expressed bacterial genes may be
used and are provided by the University of Wisconsin
15 Package Version 9.0, Genetics Computer Group, Madison,
WI. Other useful codon frequency tables include
"Celegans_high.cod", "Celegans_low.cod",
20 "Drosophila_high.cod", "Human_high.cod",
"Maize_high.cod", and "Yeast_high.cod".

In other embodiments, nucleic acid molecules
25 encode beta secretase variants with conservative amino
acid substitutions as defined above, beta secretase
variants comprising an addition and/or a deletion of
one or more N-linked or O-linked glycosylation sites,
30 or beta secretase polypeptide fragments as described
above. In addition, nucleic acid molecules may encode
any combination of beta secretase variants, fragments,
20 and fusion polypeptides described herein.

Preferred nucleic acid molecule fragments
35 encode the following fragments of SEQ ID NO:4: amino
acids 45-501; amino acids 46-501; amino acids 46-460;
amino acids 45-460; amino acids 1-460; amino acids 93-
25 292; amino acids 93-293; amino acids 91-295; amino
acids 90-295; amino acids 90-300; amino acids 62-420;
amino acids 1-420; amino acids 62-460; amino acids 90-
40 460; amino acids 62-501; amino acids 62-460; amino
acids 90-293; amino acids 90-300; amino acids 1-420;
30 amino acids 46-420; amino acids 62-420; amino acids 73-
420; amino acids 83-420; amino acids 90-420; amino
acids 62-417; amino acids 73-417; amino acids 83-417;
45 amino acids 90-417; amino acids 62-410; amino acids 73-
410; amino acids 83-410; amino acids 90-410; amino
35 acids 62-402; amino acids 73-402; amino acids 83-402;
and amino acids 90-402.

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- 25 -

10 Additionally preferred nucleic acid molecule
fragments include nucleic acid molecules encoding each
of the foregoing fragments joined to a nucleic acid
molecule encoding a peptide or polypeptide such as, for
5 example, the Fc portion of human IgG.

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Vectors and Host Cells

20 A nucleic acid molecule encoding a beta
secretase polypeptide can be inserted into an
10 appropriate expression vector in order to generate beta
secretase polypeptides. The vector is typically
selected to be functional in the particular host cell
employed (i.e., the vector is compatible with the host
cell machinery such that amplification of the gene
25 and/or expression of the gene can occur). A nucleic
acid molecule encoding a beta secretase polypeptide may
be amplified/expressed in prokaryotic, yeast, insect
(baculovirus systems) and/or eukaryotic host cells.
30 Selection of the host cell will depend in part on
20 whether an beta secretase polypeptide is to be post-
translationally modified (e.g., glycosylated and/or
phosphorylated). If so, yeast, insect, or mammalian
host cells are preferable.

35 Typically, expression vectors used in any of
25 the host cells will contain sequences for plasmid
maintenance and for cloning and expression of exogenous
nucleotide sequences. Such sequences, collectively
40 referred to as "flanking sequences" will typically
include one or more of the following nucleic acid
30 molecules: a promoter, one or more enhancer sequences,
an origin of replication, a transcriptional termination
sequence, a complete intron sequence containing a donor
and acceptor splice site, a leader sequence for
secretion, a ribosome binding site, a polyadenylation
45 sequence, a polylinker region for inserting the nucleic
50 acid encoding the polypeptide to be expressed, and a

selectable marker element. Each of these sequences is
discussed below.

Optionally, the vector may contain a "tag" sequence, i.e., an oligonucleotide molecule located at 5 the 5' or 3' end of the beta secretase polypeptide coding sequence; the oligonucleotide molecule encodes polyHis (such as hexaHis), or other "tag" such as FLAG, HA (hemagglutinin Influenza virus) or myc for which commercially available antibodies exist. This tag is 10 typically fused to the polypeptide upon expression of the polypeptide, and can serve as a means for affinity purification of the beta secretase polypeptide from the host cell. Affinity purification can be accomplished, for example, by column chromatography using antibodies 15 against the tag as an affinity matrix. Optionally, the tag can subsequently be removed from the purified beta secretase polypeptide by various means such as using one or more selected peptidases for cleavage.

Flanking sequences may be homologous (i.e., 30 from the same species and/or strain as the host cell), heterologous (i.e., from a species other than the host cell species or strain), hybrids (i.e., a combination 35 of flanking sequences from more than one source), or synthetic, or native sequences which normally function 40 to regulate beta secretase expression. As such, the source of flanking sequences may be any prokaryotic or eukaryotic organism, any vertebrate or invertebrate organism, or any plant, provided that the flanking sequence is(are) functional in, and can be activated 45 by, the host cell machinery.

The flanking sequences useful in the vectors 45 of this invention may be obtained by any of several methods well known in the art. Typically, flanking sequences useful herein other than the beta secretase 50 gene flanking sequences will have been previously identified by mapping and/or by restriction

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- 27 -

10 endonuclease digestion and can thus be isolated from
the proper tissue source using the appropriate
restriction endonucleases. In some cases, the full
nucleotide sequence of one or more flanking sequence
5 may be known. Here, the flanking sequence may be
synthesized using the methods described above for
nucleic acid synthesis or cloning.

15 20 Where all or only a portion of the flanking
sequence is known, it may be obtained using PCR and/or
by screening a genomic library with suitable
oligonucleotide and/or flanking sequence fragments from
the same or another species.

25 30 35 Where the flanking sequence is not known, a
fragment of DNA containing a flanking sequence may be
isolated from a larger piece of DNA that may contain,
for example, a coding sequence or even another gene or
genes. Isolation may be accomplished by restriction
endonuclease digestion to produce the proper DNA
fragment followed by isolation using agarose gel
purification, Qiagen® column chromatography, or other
method known to the skilled artisan. Selection of
suitable enzymes to accomplish this purpose will be
readily apparent to one of ordinary skill in the art.

40 45 An origin of replication is typically a part
25 of prokaryotic expression vectors purchased
commercially, and aids in the amplification of the
vector in a host cell. Amplification of the vector to
a certain copy number can, in some cases, be important
for optimal expression of the beta secretase
30 polypeptide. If the vector of choice does not contain
an origin of replication site, one may be chemically
synthesized based on a known sequence, and ligated into
the vector.

50 55 The origin of replication from the plasmid
35 pBR322 (Product No. 303-3S, New England Biolabs,
Beverly, MA) is suitable for most Gram-negative

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- 28 -

bacteria. Various origin of replication elements (e.g., SV40, polyoma, adenovirus, vesicular stomatitis virus (VSV) or papillomaviruses such as HPV or BPV) are useful for cloning vectors in mammalian cells.

10 5 Generally, the origin of replication component is not needed for mammalian expression vectors (for example, the SV40 origin is often used only because it contains the early promoter).

15 10 A transcription termination sequence is typically located 3' of the end of a polypeptide coding regions and serves to terminate transcription.

20 25 Usually, a transcription termination sequence in prokaryotic cells is a G-C rich fragment followed by a poly T sequence. While the sequence is easily cloned from a library or even purchased commercially as part of a vector, it can also be readily synthesized using methods for nucleic acid synthesis such as those described above.

30 30 A selectable marker gene element encodes a protein necessary for the survival and growth of a host cell grown in a selective culture medium. Typical selection marker genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, tetracycline, or kanamycin for prokaryotic 35 40 host cells, (b) complement auxotrophic deficiencies of the cell; or (c) supply critical nutrients not available from complex media. Preferred selectable markers are the kanamycin resistance gene, the ampicillin resistance gene, and the tetracycline 45 50 resistance gene.

45 Other selection genes may be used to amplify the gene which will be expressed. Amplification is the process wherein genes which are in greater demand for the production of a protein critical for growth are 50 55 reiterated in tandem within the chromosomes of successive generations of recombinant cells. Examples

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- 29 -

of suitable selectable markers for mammalian cells
10 include dihydrofolate reductase (DHFR) and thymidine
kinase. The mammalian cell transformants are placed
under selection pressure which only the transformants
15 are uniquely adapted to survive by virtue of the marker
present in the vector. Selection pressure is imposed
by culturing the transformed cells under conditions in
which the concentration of selection agent in the
medium is successively changed, thereby leading to
20 amplification of both the selection gene and the DNA
that encodes beta secretase. As a result, increased
quantities of beta secretase are synthesized from the
amplified DNA.

A ribosome binding site is usually necessary
25 for translation initiation of mRNA and is characterized
15 by a Shine-Dalgarno sequence (prokaryotes) or a Kozak
sequence (eukaryotes). The element is typically
located 3' to the promoter and 5' to the coding
30 sequence of the beta secretase polypeptide to be
expressed. The Shine-Dalgarno sequence is varied but
20 is typically a polypurine (i.e., having a high A-G
content). Many Shine-Dalgarno sequences have been
35 identified, each of which can be readily synthesized
using methods set forth above and used in a prokaryotic
25 vector.

A leader, or signal, sequence may be used to
40 direct an beta secretase polypeptide out of the host
cell. Typically, the signal sequence is positioned in
the coding region of the beta secretase nucleic acid
30 molecule, or directly at the 5' end of the beta
secretase polypeptide coding region.

The signal sequence may be a component of the
45 vector, or it may be a part of beta secretase DNA that
is inserted into the vector. The native beta secretase
35 DNA encodes a signal sequence at the amino terminus of
50 the protein that is cleaved during post-translational

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- 30 -

processing of the molecule to form the mature beta
secretase protein product.

Included within the scope of this invention
are beta secretase nucleic acid molecules with the

- 5 native signal sequence as well as beta secretase
nucleic acid molecules wherein the native signal
sequence is deleted and replaced with a heterologous
signal sequence. The heterologous signal sequence
selected should be one that is recognized and
10 processed, i.e., cleaved by a signal peptidase, by the
host cell. For expression of beta secretase in
prokaryotic host cells, the native signal sequence is
typically replace by a prokaryotic signal sequence
selected, for example, from the group of the alkaline
15 phosphatase, penicillinase, or heat-stable enterotoxin
II leaders. For beta secretase expression in yeast
host cells, the native beta secretase signal sequence
may be substituted by any of the yeast invertase, alpha
factor, or acid phosphatase signal sequences. For beta
20 secretase expression in mammalian host cells, the
native beta secretase signal sequence is satisfactory,
although other mammalian signal sequences may be
suitable.

In many cases, transcription of a nucleic
acid molecule is increased by the presence of one or
more introns in the vector; this is particularly true
where a polypeptide is produced in eukaryotic host
cells, especially mammalian host cells. The introns
used may be naturally occurring within the beta
25 secretase gene, especially where the gene used is a
full length genomic sequence or a fragment thereof.
Where the intron is not naturally occurring within the
gene (as for most cDNAs), the intron(s) may be obtained
from another source. The position of the intron with
30 respect to flanking sequences and the beta secretase
gene is generally important, as the intron must be
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- 31 -

transcribed to be effective. Thus, when an beta
secretase cDNA molecule is being expressed, the
preferred position for the intron is 3' to the
transcription start site, and 5' to the polyA
transcription termination sequence. Preferably, the
intron or introns will be located on one side or the
other (i.e., 5' or 3') of the cDNA such that it does
not interrupt the coding sequence. Any intron from any
source, including any viral, prokaryotic and eukaryotic
(plant or animal) organisms, may be used to practice
this invention, provided that it is compatible with the
host cell(s) into which it is inserted. Also included
herein are synthetic introns. Optionally, more than
one intron may be used in the vector.

The expression and cloning vectors of the
present invention will typically contain a promoter
that is recognized by the host organism and operably
linked to the molecule encoding the beta secretase
polypeptide. Promoters are untranslated nucleic acid
molecules located upstream(5') to the start codon of a
structural gene (generally within about 100 to 1000 bp)
that control the transcription and translation of a
particular molecule, such as the beta secretase gene.
Promoters are conventionally grouped into one of two
classes, inducible promoters and constitutive
promoters. Inducible promoters initiate increased
levels of transcription of the structural gene in
response to some change in culture conditions, such as
the presence or absence of a nutrient or a change in
temperature.

A large number of promoters that are
functional in various host cells are well known and
readily available. These promoters can be operably
linked to the DNA encoding beta secretase by removing
the native promoter from the beta secretase gene (via
restriction enzyme digestion) and inserting the desired

10 promoter into the vector. While the native beta
secrctase promoter sequence may be used to direct
amplification and/or expression of the beta secretase
gene in mammalian cells, a heterologous promoter is
5 preferred if it permits greater transcription and
higher yields of beta secretase polypeptide as compared
to the native beta secretase promoter, and if it is
compatible with the host cell system that has been
15 selected for use.

20 Promoters suitable for use with prokaryotic
hosts include, without limitation, the beta-lactamase
and lactose promoter systems; alkaline phosphatase, a
tryptophan (trp) promoter system; and hybrid promoters
such as the tac promoter. Other known bacterial
25 promoters are also suitable. Their sequences have been
published, thereby enabling one skilled in the art to
ligate them to the desired DNA sequence(s), using
linkers or adapters as needed to supply any required
30 restriction sites.

35 Suitable promoter sequences for use with
yeast host cells are also well known in the art. Yeast
enhancers are advantageously used with yeast promoters.
Suitable promoters for use with mammalian host cells
are well known and include those obtained from the
40 genomes of viruses such as polyoma virus, fowlpox
virus, adenovirus (such as Adenovirus 2), bovine
papilloma virus, avian sarcoma virus, cytomegalovirus,
a retrovirus, hepatitis-B virus and most preferably
Simian Virus 40 (SV40).

45 Suitable mammalian host cell promoters
include heterologous mammalian promoters, e.g., heat-
shock promoters and the actin promoter.

50 Additional promoters which may be of interest
in controlling beta secretase expression include, but
35 are not limited to: the SV40 early promoter region;
the CMV promoter; the promoter contained in the 3' long

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- 33 -

terminal repeat of Rous sarcoma virus; the herpes
10 thymidine kinase promoter; the regulatory sequences of
the metallothioneine gene; prokaryotic expression
vectors such as the beta-lactamase promoter; or the tac
5 promoter. Also of interest are the following animal
transcriptional control regions, which exhibit tissue
specificity and have been utilized in transgenic
15 animals: the insulin gene control region which is
active in pancreatic beta cells; the immunoglobulin
gene control region which is active in lymphoid cells;
20 the mouse mammary tumor virus control region which is
active in testicular, breast, lymphoid and mast cells;
albumin gene control region which is active in liver;
the alphafetoprotein gene control region which is
25 active in liver; the alpha 1-antitrypsin gene control
region which is active in the liver; the beta-globin
gene control region which is active in myeloid cells;
the myelin basic protein gene control region which is
30 active in oligodendrocyte cells in the brain; the
myosin light chain-2 gene control region which is
active in skeletal muscle; and the gonadotropic
releasing hormone gene control region which is active
35 in the hypothalamus.

Another optional component of the vector used
40 for beta secretase polypeptide expression is the
enhancer element. This nucleic acid molecule may be
inserted into the vector to increase the transcription
in higher eukaryotic host cells of a genomic DNA or
cDNA molecule encoding beta secretase polypeptide.
45 Enhancers are usually about 10-300 nucleotides in
length and act on the promoter to increase its
transcription. Several enhancer sequences available
from mammalian genes are known (e.g., globin, elastase,
albumin, alpha-feto-protein and insulin). Typically,
50 however, an enhancer from a virus will be used. The
SV40 enhancer, the cytomegalovirus early promoter

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- 34 -

enhancer, the polyoma enhancer, and adenovirus
10 enhancers are exemplary enhancers useful for the
activation of eukaryotic promoters. While an enhancer
may be inserted into the vector either 5' or 3' to the
5 beta secretase gene or cDNA, it is typically located at
a site 5' from the promoter.

15 Expression vectors of the invention may be
constructed from a starting vector such as a
commercially available vector. Such vectors may or may
not contain all of the desired flanking sequences.
20 Where one or more of the flanking sequences set forth
above are not already present in the vector to be used,
they may be individually obtained and ligated into the
vector. Methods used for obtaining each of the
25 flanking sequences are well known to one skilled in the
art.

30 Preferred vectors for practicing this
invention are those which are compatible with
bacterial, insect, and mammalian host cells. Such
35 vectors include, inter alia, pCRII, pCR3, and pcDNA3.1
(Invitrogen Company, San Diego, CA), PBSII (Stratagene
Company, La Jolla, CA), pET15b (Novagen, Madison, WI),
pGEX (Pharmacia Biotech, Piscataway, NJ), pEGFP-N2
(Clontech, Palo Alto, CA), pETL (BlueBacII;
40 Invitrogen), and pFastBacDual (Gibco/BRL, Grand Island,
NY).

45 Additional possible vectors include, but are
not limited to, cosmids, plasmids or modified viruses,
but the vector system must be compatible with the
selected host cell. Such vectors include, but are not
50 limited to plasmids such as Bluescript[®] plasmid
derivatives (a high copy number ColE1-based phagemid,
Stratagene Cloning Systems Inc., La Jolla CA), PCR
cloning plasmids designed for cloning Taq-amplified PCR
products (e.g., TOPO[™] TA Cloning[®] Kit, PCR2.1[™] plasmid

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- 35 -

10 derivatives, Invitrogen, Carlsbad, CA), and mammalian, yeast or virus vectors such as a baculovirus expression system (pBacPAK plasmid derivatives, Clontech, Palo Alto, CA). The recombinant molecules can be introduced
5 into host cells via transformation, transfection, infection, electroporation, or other known techniques.

15 After the vector has been constructed and a nucleic acid molecule encoding an beta secretase polypeptide has been inserted into the proper site of
10 the vector, the completed vector may be inserted into a suitable host cell for amplification and/or polypeptide expression.

20 Host cells may be prokaryotic host cells (such as *E. coli*) or eukaryotic host cells (such as a
25 yeast cell, an insect cell, or a vertebrate cell). The host cell, when cultured under appropriate conditions, synthesizes an beta secretase polypeptide which can subsequently be collected from the culture medium (if the host cell secretes it into the medium) or directly
30 from the host cell producing it (if it is not secreted). Selection of an appropriate host cell will depend upon various factors, such as desired expression levels, polypeptide modifications that are necessary
35 for activity, such as glycosylation or phosphorylation,
25 and ease of folding into a biologically active molecule.

40 Suitable host cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO), human embryonic kidney (HEK) 293 or 293T cells,
45 30 or 3T3 cells. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. Other suitable mammalian cell lines, are the monkey COS-1 and COS-7
50 35 cell lines, and the CV-1 cell line. Further exemplary mammalian host cells include primate cell lines and

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- 36 -

rodent cell lines, including transformed cell lines. 10 Normal diploid cells, cell strains derived from *in vitro* culture of primary tissue, as well as primary explants, are also suitable. Candidate cells may be 5 genotypically deficient in the selection gene, or may contain a dominantly acting selection gene. Other suitable mammalian cell lines include but are not limited to, mouse neuroblastoma N2A cells, HeLa, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or 10 NIH mice, BHK or HaK hamster cell lines. Each of these cell lines is known by and available to those skilled 20 in the art of protein expression.

Similarly useful as host cells suitable for the present invention are bacterial cells. For 25 example, the various strains of *E. coli* (e.g., HB101, DH5a, DH10, and MC1061 are well-known as host cells in the field of biotechnology. Various strains of *B. subtilis*, *Pseudomonas spp.*, other *Bacillus spp.*, 30 *Streptomyces spp.*, and the like may also be employed in 20 this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present 35 invention. Preferred yeast cells include, for example, 25 *Saccharomyces cerevisiae*.

Additionally, where desired, insect cell 40 systems may be utilized in the methods of the present invention. Such systems are described for example in Kitts et al. (*Biotechniques*, 14:810-817 [1993]), 45 30 Lucklow (*Curr. Opin. Biotechnol.*, 4:564-572 [1993]) and Lucklow et al. (*J. Virol.*, 67:4566-4579 [1993]). Preferred insect cells are Sf-9 and Hi5 (Invitrogen, Carlsbad, CA).

Transformation or transfection of an 50 35 expression vector for an beta secretase polypeptide into a selected host cell may be accomplished by well

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- 37 -

known methods including methods such as calcium chloride, electroporation, microinjection, lipofection or the DEAE-dextran method. The method selected will in part be a function of the type of host cell to be used. These methods and other suitable methods are well known to the skilled artisan, and are set forth, for example, in Sambrook et al., *supra*.

Polypeptide Production

Host cells comprising a beta secretase expression vector may be cultured using standard media well known to the skilled artisan. The media will usually contain all nutrients necessary for the growth and survival of the cells. Suitable media for culturing *E. coli* cells are for example, Luria Broth (LB) and/or Terrific Broth (TB). Suitable media for culturing eukaryotic cells are RPMI 1640, MEM, DMEM, all of which may be supplemented with serum and/or growth factors as required by the particular cell line being cultured. A suitable medium for insect cultures is Grace's medium supplemented with yeastolate, lactalbumin hydrolysate, and/or fetal calf serum as necessary.

Typically, an antibiotic or other compound useful for selective growth of transfected or transformed cells is added as a supplement to the media. The compound to be used will be dictated by the selectable marker element present on the plasmid with which the host cell was transformed. For example, where the selectable marker element is kanamycin resistance, the compound added to the culture medium will be kanamycin.

The amount of a beta secretase polypeptide produced by a host cell can be evaluated using standard methods known in the art. Such methods include, without limitation, Western blot analysis,

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- 38 -

10 SDS-polyacrylamide gel electrophoresis, non-denaturing
gel electrophoresis, HPLC separation,
immunoprecipitation, and/or activity assays such as DNA
binding gel shift assays.

15 5 If a beta secretase polypeptide has been
designed to be secreted from the host cells, the
majority of polypeptide may be found in the cell
culture medium. If however, the beta secretase
polypeptide is not secreted from the host cells, it
20 10 will be present in the cytoplasm and/or the nucleus
(for eukaryotic host cells) or in the cytosol (for gram
negative bacteria host cells).

25 15 For a beta secretase polypeptide situated in
the host cell cytoplasm and/or nucleus, the host cells
are typically first disrupted mechanically or with
detergent to release the intra-cellular contents into a
buffered solution. Beta secretase polypeptide can then
be isolated from this solution.

30 20 Purification of a beta secretase polypeptide
from solution can be accomplished using a variety of
techniques. If the polypeptide has been synthesized
such that it contains a tag such as hexahistidine or
other small peptide such as FLAG (Eastman Kodak Co.,
35 25 New Haven, CT) or myc (Invitrogen, Carlsbad, CA) at
either its carboxyl or amino terminus, it may
essentially be purified in a one-step process by
passing the solution through an affinity column where
the column matrix has a high affinity for the tag or
40 30 for the polypeptide directly (i.e., a monoclonal
antibody specifically recognizing beta secretase
polypeptide). For example, polyhistidine binds with
great affinity and specificity to nickel, thus an
affinity column of nickel (such as the Qiagen® nickel
45 35 columns) can be used for purification of beta secretase
polypeptide/polyHis. (See for example, Ausubel et al.,

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- 39 -

eds., *Current Protocols in Molecular Biology, Section 10.11.8*, John Wiley & Sons, New York [1993]).

Where a beta secretase polypeptide is prepared without a tag attached and no antibodies are available, other well known procedures for purification can be used. Such procedures include, without limitation, ion exchange chromatography, molecular sieve chromatography, HPLC, native gel electrophoresis in combination with gel elution, and preparative isoelectric focusing ("Isoprime" machine/technique, Hoefer Scientific). In some cases, two or more of these techniques may be combined to achieve increased purity.

If a beta secretase polypeptide is produced intracellularly, the intracellular material (including inclusion bodies for gram-negative bacteria) can be extracted from the host cell using any standard technique known to the skilled artisan. For example, the host cells can be lysed to release the contents of the periplasm/cytoplasm by French press, homogenization, and/or sonication followed by centrifugation.

If a beta secretase polypeptide has formed inclusion bodies in the cytosol, the inclusion bodies can often bind to the inner and/or outer cellular membranes and thus will be found primarily in the pellet material after centrifugation. The pellet material can then be treated at pH extremes or with chaotropic agent such as a detergent, guanidine, guanidine derivatives, urea, or urea derivatives in the presence of a reducing agent such as dithiothreitol at alkaline pH or tris carboxyethyl phosphine at acid pH to release, break apart, and solubilize the inclusion bodies. The beta secretase polypeptide in its now soluble form can then be analyzed using gel electrophoresis, immunoprecipitation or the like. If

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it is desired to isolate the beta secretase polypeptide, isolation may be accomplished using standard methods such as those set forth below and in Marston et al. (Meth. Enz., 182:264-275 [1990]).

- 10 5 In some cases, a beta secretase polypeptide may not be biologically active upon isolation. Various methods for "refolding" or converting the polypeptide to its tertiary structure and generating disulfide linkages, can be used to restore biological activity.
- 15 10 Such methods include exposing the solubilized polypeptide to a pH usually above 7 and in the presence of a particular concentration of a chaotropic agent. In most cases the refolding/oxidation solution will also contain a reducing agent or a reducing agent plus 20 15 its oxidized form in a specific ratio to generate a particular redox potential allowing for disulfide shuffling to occur in the formation of cysteine bridge(s) of the polypeptide. Some commonly used redox couples include cysteine/cystamine, glutathione 25 20 (GSH)/dithiobis GSH, cupric chloride, dithiothreitol(DTT)/dithiane DTT, 2-mercaptopropanoic acid (bME)/dithio-b(ME). In many instances a cosolvent is necessary to increase the efficiency of 30 35 the refolding and the more common reagents used for this purpose include glycerol, polyethylene glycol of various molecular weights, arginine and the like.

40 45 If inclusion bodies are not formed to a significant degree upon expression of an beta secretase polypeptide, the polypeptide will be found primarily in 30 35 the supernatant after centrifugation of the cell homogenate and may be further isolated from the supernatant using methods such as those set forth below.

50 55 In situations where it is preferable to partially or completely purify an beta secretase polypeptide such that it is partially or substantially

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- 41 -

- free of contaminants, standard methods known to the one skilled in the art may be used. Such methods include, without limitation, separation by electrophoresis followed by electroelution, various types of chromatography (affinity, immunoaffinity, molecular sieve, and/or ion exchange), and/or high pressure liquid chromatography. In some cases, it may be preferable to use more than one of these methods for complete purification.
- Beta secretase polypeptides, fragments, and/or derivatives thereof may also be prepared by chemical synthesis methods using techniques known in the art such as those set forth by Merrifield et al., (*J. Am. Chem. Soc.*, 85:2149 [1963]), Houghten et al. (*Proc Natl Acad. Sci. USA*, 82:5132 [1985]), and Stewart and Young (*Solid Phase Peptide Synthesis*, Pierce Chemical Co., Rockford, IL [1984]). Using these methods, beta secretase fragments of up to about 75 amino acids in length can be prepared.
- Typically, beta-secretase fragments and variants are synthesized from readily available starting materials. Synthesis is usually conducted from carboxy to amino terminus. During synthesis, the alpha-amino of the amino acid to be added is protected by a urethane such as Boc, Cbz, Fmoc, or Alloc (see Greene et al., *Protective Groups in Organic Synthesis*, 2d. ed., John Wiley and Sons [1991] for a list of protective groups) while the free carboxyl is activated with an activating reagent which is usually a carbodiimide such as DCC (Dicyclohexyl carbodiimide), EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide), or DIC (diisopropylcarbodiimide). A preferred protective group is Fmoc. The activating reagent can optionally be used in the presence of a catalyst such as Hobt (N-Hydroxybenzotriazole) Hoat (7-aza-N-

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- 42 -

hydroxybenzotriazole), Hosu, or Dmap
10 (Dimethylaminopyridine). After the peptide is
completely synthesized, the side chain protecting
groups may be removed using methods set forth in the
5 above cited references. Such methods include,
without limitation, hydrogenation in the presence of
15 a catalyst such as palladium, platinum, or rhodium;
treatment with sodium in liquid ammonia,
hydrochloric, hydrofluoric, hydrobromic, formic,
20 trifluoromethanesulfonic, or trifluoroacetic acid;
secondary amines; fluoride ion; trimethylsilyl
halides such as bromide and iodide; or alkali.

The above described methods may be
accomplished manually or using an automated peptide
25 synthesizer such as an Applied Biosystems model 430,
430A, A431, or A433, using programming modules as
defined by the manufacturer's manuals.

To generate beta secretase fragments or
variants using chemical synthesis that are longer
30 than about 75 amino acids, a technique known as
chemical ligation can be used in which peptides can
be ligated together. This technique is described by
35 Baca et al. (*J. Amer. Chem. Soc.*, 117:1881-1887
[1995]) and by Schröder et al. (*Int. J. Peptide
Protein Res.*, 40:180-193 [1992]).

Chemically synthesized beta-secretase
40 polypeptides, fragments, and variants may be
oxidized to permit the formation of disulfide
bridges using standard methods set forth in the
30 above cited references.

The beta-secretase polypeptides or
45 fragments thereof are expected to have biological
activity comparable to beta-secretase polypeptides
or fragments thereof produced recombinantly and thus
35 may be used interchangeably with recombinant or
beta-secretase peptide.

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- 43 -

Another means of obtaining beta secretase polypeptide is via purification from biological samples such as source tissues and/or fluids in which the beta secretase polypeptide is naturally found. Such purification can be conducted using methods for protein purification as described above. The presence of the beta secretase polypeptide during purification may be monitored using, for example, an antibody prepared against recombinantly produced beta secretase polypeptide or peptide fragments thereof.

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Polypeptides

Polypeptides of the invention include isolated beta secretase polypeptides and polypeptides related thereto including fragments, variants, fusion polypeptides, and derivatives as defined hereinabove.

Beta secretase fragments of the invention may result from truncations at the amino terminus (with or without a leader sequence), truncations at the carboxy terminus, and/or deletions internal to the polypeptide. In preferred embodiments, truncations and/or deletions comprise about 10 amino acids, or about 20 amino acid, or about 50 amino acids, or about 75 amino acids, or about 100 amino acids. The polypeptide fragments so produced will comprise about 25 contiguous amino acids, or about 50 amino acids, or about 75 amino acids, or about 100 amino acids, or about 150 amino acids, or about 200 amino acids. Such beta secretase polypeptides fragments may optionally comprise an amino terminal methionine residue.

Preferred beta secretase polypeptide fragments of SEQ ID NO: 4 include: amino acids 45-501; amino acids 46-501; amino acids 46-460; amino acids 45-460; amino acids 1-460; amino acids 93-292; amino acids 93-293; amino acids 91-295; amino acids 90-295; amino

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- 44 -

10 acids 90-300; amino acids 62-420; amino acids 1-420;
 amino acids 62-460; amino acids 90-460; amino acids 62-
 501; amino acids 62-460; amino acids 90-293; amino
 acids 90-300; amino acids 1-420; amino acids 46-420;
5 amino acids 62-420; amino acids 73-420; amino acids 83-
 420; amino acids 90-420; amino acids 62-417; amino
 acids 73-417; amino acids 83-417; amino acids 90-417;
 amino acids 62-410; amino acids 73-410; amino acids 83-
 410; amino acids 90-410; amino acids 62-402; amino
10 acids 73-402; amino acids 83-402; and amino acids 90-
 402.

20 Additionally preferred fragments include each
 of the foregoing prepared as a fusion peptide with a
 second peptide or polypeptide such as, for example, the
25 Fc portion of human IgG..

30 Beta secretase polypeptide variants of the
 invention include one or more amino acid substitutions,
 additions and/or deletions as compared to any of SEQ ID
 NOS: 4, 5, and 6. In preferred embodiments, the
35 20 variants have from 1 to 3, or from 1 to 5, or from 1 to
 10, or from 1 to 15, or from 1 to 20, or from 1 to 25,
 or from 1 to 50, or from 1 to 75, or from 1 to 100, or
 more than 100 amino acid substitutions, insertions,
 additions and/or deletions, wherein the substitutions
 25 may be conservative, as defined above, or non-
 conservative or any combination thereof. The variants
 40 may have additions of amino acid residues either at the
 carboxy terminus or at the amino terminus (with or
 without a leader sequence).

45 30 Preferred beta secretase polypeptide variants
 include glycosylation variants wherein the number
 and/or type of glycosylation sites has been altered
 compared to native beta secretase polypeptide. In one
 35 embodiment, beta secretase variants comprise a greater
 or a lesser number of N-linked glycosylation sites. An
 50 N-linked glycosylation site is characterized by the

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- 45 -

sequence: Asn-X-Ser or Thr, where the amino acid residue designated as X may be any type of amino acid except proline. Substitution(s) of amino acid residues to create this sequence provides a potential new site 5 for addition of an N-linked carbohydrate chain. Alternatively, substitutions to eliminate this sequence will remove an existing N-linked carbohydrate chain. Also provided is a rearrangement of N-linked carbohydrate chains wherein one or more N-linked 10 glycosylation sites (typically those that are naturally occurring) are eliminated and one or more new N-linked 20 sites are created.

Beta secretase fusion polypeptides of the invention comprise beta secretase polypeptides, 25 fragments, variants, or derivatives fused to a heterologous peptide or protein. Heterologous peptides and proteins include, but are not limited to, an epitope to allow for detection and/or isolation of an 30 beta secretase fusion polypeptide, a transmembrane 20 receptor protein or a portion thereof, such as an extracellular domain, or a transmembrane and intracellular domain, a ligand or a portion thereof which binds to a transmembrane receptor protein, an 35 enzyme or portion thereof which is catalytically active, a protein or peptide which promotes 25 oligomerization, such as leucine zipper domain, and a protein or peptide which increase stability, such as an immunoglobulin constant region. A beta secretase 40 polypeptide may be fused to itself or to a fragment, 30 variant, or derivative thereof. Fusions may be made either at the amino terminus or at the carboxy terminus 45 of an beta secretase polypeptide, and may be direct with no linker or adapter molecule or may be through a linker or adapter molecule, such as one or more amino 50 acid residues up to about 20 amino acids residues, or up to about 50 amino acid residues. A linker or

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- 46 -

10 adapter molecule may also be designed with a cleavage site for a DNA restriction endonuclease or for a protease to allow for separation of the fused moieties.

15 In a preferred embodiment, a beta secretase 5 polypeptide, fragment, variant and/or derivative is fused to an Fc region of human IgG. In one example, a human IgG hinge, CH₂ and CH₃ region may be fused at either the N-terminus or C-terminus of the beta secretase polypeptides using methods known to the
20 skilled artisan. In another example, a portion of a hinge regions and CH₂ and CH₃ regions may be fused. The beta secretase Fc-fusion polypeptide so produced may be purified by use of a Protein A affinity column.
25 In addition, peptides and proteins fused to an Fc 15 region have been found to exhibit a substantially greater half-life *in vivo* than the unfused counterpart. Also, a fusion to an Fc region allows for dimerization/multimerization of the fusion polypeptide.

30 Beta secretase polypeptide derivatives are 20 included in the scope of the present invention. Such derivatives are chemically modified beta secretase 35 polypeptide compositions in which beta secretase polypeptide is linked to a polymer. The polymer selected is typically water soluble so that the protein 40 to which it is attached does not precipitate in an aqueous environment, such as a physiological 45 environment. The polymer selected is usually modified to have a single reactive group, such as an active ester for acylation or an aldehyde for alkylation, so 30 that the degree of polymerization may be controlled as provided for in the present methods. The polymer may be of any molecular weight, and may be branched or unbranched. Included within the scope of beta secretase polypeptide polymers is a mixture of 35 polymers. Preferably, for therapeutic use of the end-

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- 47 -

10 product preparation, the polymer will be pharmaceutically acceptable.

15 The water soluble polymer or mixture thereof may be selected from the group consisting of, for example, polyethylene glycol (PEG), monomethoxy-polyethylene glycol, dextran, cellulose, or other carbohydrate based polymers, poly-(N-vinyl pyrrolidone) polyethylene glycol, propylene glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, 20 polyoxyethylated polyols (e.g., glycerol) and polyvinyl alcohol.

25 For the acylation reactions, the polymer(s) selected should have a single reactive ester group.

30 For reductive alkylation, the polymer(s) selected should have a single reactive aldehyde group. A preferred reactive aldehyde is polyethylene glycol propionaldehyde, which is water stable, or mono C1-C10 alkoxy or aryloxy derivatives thereof (see U.S. Patent No. 5,252,714).

35 Pegylation of beta secretase polypeptides may be carried out by any of the pegylation reactions known in the art, as described for example in the following references: *Focus on Growth Factors* 3: 4-10 (1992); EP 0 154 316; and EP 0 401 384. Preferably, the 40 pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous reactive water-soluble polymer) as described below.

45 A particularly preferred water-soluble polymer for use herein is polyethylene glycol, abbreviated PEG. As used herein, polyethylene glycol is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono- (C1-C10) alkoxy- or aryloxy-polyethylene glycol.

50 In general, chemical derivatization may be performed under any suitable conditions used to react a

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- 48 -

10 biologically active substance with an activated polymer
molecule. Methods for preparing pegylated beta
secretase polypeptides will generally comprise the
steps of (a) reacting the polypeptide with polyethylene
5 glycol (such as a reactive ester or aldehyde derivative
of PEG) under conditions whereby beta secretase
polypeptide becomes attached to one or more PEG groups,
and (b) obtaining the reaction product(s). In general,
the optimal reaction conditions for the acylation
10 reactions will be determined based on known parameters
and the desired result. For example, the larger the
ratio of PEG: protein, the greater the percentage of
poly-pegylated product.

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25 In a preferred embodiment, the beta secretase
polypeptide derivative will have a single PEG moiety at
the N terminus.

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40 Generally, conditions which may be alleviated
or modulated by administration of the present beta
secretase polypeptide derivative include those
described herein for beta secretase polypeptides.
However, the beta secretase polypeptide derivative
disclosed herein may have additional activities,
enhanced or reduced biological activity, or other
characteristics, such as increased or decreased half-
25 life, as compared to the non-derivatized molecules.

Antibodies

45 Beta secretase polypeptides, fragments,
variants and derivatives may be used to prepare
30 antibodies using methods known in the art. Thus,
antibodies and antibody fragments that bind beta
secretase polypeptides are within the scope of the
present invention. Antibodies may be polyclonal,
monoclonal, recombinant, chimeric, humanized, fully
35 human, single chain and/or bispecific.

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- 49 -

- 10 Polyclonal antibodies directed toward a beta secretase polypeptide generally are raised in animals (rabbits or mice) by multiple subcutaneous or intraperitoneal injections of beta secretase in
15 combination with an adjuvant. It may be useful to conjugate a beta secretase polypeptide, or a variant, fragment or derivative thereof to a carrier protein that is immunogenic in the species to be immunized, such as keyhole limpet heocyanin, serum, albumin,
20 bovine thyroglobulin, or soybean trypsin inhibitor. Also, aggregating agents such as alum are used to enhance the immune response. After immunization, the animals can be bled and the serum is assayed for anti-beta secretase antibody titer.
- 25 15 Monoclonal antibodies directed toward beta secretase polypeptide can be produced using any method which provides for the production of antibody molecules by continuous cell lines in culture. Examples of suitable methods for preparing monoclonal antibodies
30 20 include hybridoma method of Kohler et al., *Nature* 256: 495-497 (1975), and the human B-cell hybridoma method, Kozbor, *J. Immunol.* 133: 3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 35 25 1987).
- 40 Also provided by the invention are hybridoma cell lines which produce monoclonal antibodies reactive with beta secretase polypeptides.
- 45 30 Monoclonal antibodies of the invention may be modified for use as therapeutics. One embodiment is a "chimeric" antibody in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequence in antibodies derived from a particular species or belonging to a particular
50 35 40 antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to

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corresponding sequence in antibodies derived from
another species or belonging to another antibody class
or subclass, as well as fragments of such antibodies,
so long as they exhibit the desired biological activity
5 (see U.S. Patent No. 4,816,567; and Morrison et al.,
Proc. Natl. Acad. Sci. 81, 6851-6855 [1985]).

Also included in the scope of the present
invention are monoclonal antibodies that are
"humanized". Methods for humanizing non-human
10 antibodies are well known in the art. Humanization can
be performed following methods known in the art (see
Jones et al., *Nature* 321: 522-525 [1986]; Riechmann et
al., *Nature*, 332: 323-327 [1988]; Verhoeyen et al.,
20 *Science* 239: 1534-1536 [1988]), by substituting rodent
25 complementarily-determining regions (CDRs) for the
corresponding regions of a human antibody.

Also encompassed by the invention are fully
30 human antibodies which bind beta secretase
polypeptides, fragments, variants and/or derivatives.
Such antibodies can be produced by immunization with an
35 beta secretase antigen (optionally conjugated to a
carrier) of transgenic animals (e.g., mice) that are
capable of producing a repertoire of human antibodies
in the absence of endogenous immunoglobulin production.
See, for example, Jakobovits et al., *Proc. Natl. Acad.
40 Sci.* 90: 2551-2555 (1993); Jakobovits et al., *Nature*
362: 255-258 (1993); Brugermann et al., *Year in
Immuno.* 7:33 (1993). Human antibodies can also be
45 produced in phage-display libraries (see Hoogenboom et
30 al., *J. Mol. Biol.* 227:381 [1991]; Marks et al., *J.
Mol. Biol.* 222:581 [1991]).

For diagnostic applications, anti-beta
secretase antibodies typically will be labeled with a
detectable moiety. The detectable moiety can be any
35 one which is capable of producing, either directly or
indirectly, a detectable signal. For example, the

- 51 -

detectable moiety may be a radioisotope, such as ^3H ,
10 ^{14}C , ^{32}P , ^{35}S , or ^{125}I , a fluorescent or chemiluminescent
compound, such as fluorescein isothiocyanate,
rhodamine, or luciferin; or an enzyme, such as alkaline
5 phosphatase, β -galactosidase or horseradish peroxidase.

The anti-beta secretase antibodies of the
invention may be employed in any known assay method,
such as competitive binding assays, direct and indirect
sandwich assays, and immunoprecipitation assays (see
10 Sola, *Monoclonal Antibodies: A Manual of Techniques*,
pp. 147-158 [CRC Press, Inc., 1987]) for detection and
quantitation of beta secretase polypeptides. The
antibodies will bind beta secretase polypeptides with
an affinity which is appropriate for the assay method
25 being employed.

Competitive binding assays rely on the
ability of a labeled standard (e.g., an beta secretase
polypeptide, or an immunologically reactive portion
thereof) to compete with the test sample analyte (an
20 beta secretase polypeptide) for binding with a limited
amount of anti beta secretase antibody. The amount of
an beta secretase polypeptide in the test sample is
inversely proportional to the amount of standard that
35 becomes bound to the antibodies. To facilitate
determining the amount of standard that becomes bound,
the antibodies generally are insolubilized before or
after the competition, so that the standard and analyte
that are bound to the antibodies may conveniently be
40 separated from the standard and analyte which remain
30 unbound.

Sandwich assays involve the use of two
45 antibodies, each capable of binding to a different
immunogenic portion, or epitope, of the protein to be
detected and/or quantitated. In a sandwich assay, the
35 test sample analyte is typically bound by a first

10 antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three part complex. See U.S. Patent No. 4,376,110. The second antibody may itself
5 be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assays). For example, one type of sandwich assay is an ELISA assay, in which case the
10 detectable moiety is an enzyme.

20 The anti-beta secretase antibodies of the invention also are useful for *in vivo* imaging, wherein an antibody labeled with a detectable moiety is administered to an animal, preferably into the
25 bloodstream, and the presence and location of the labeled antibody in the host is assayed. The antibody may be labeled with any moiety that is detectable in an animal, whether by nuclear magnetic resonance, radiology, or other detection means known in the art.
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20 Antibodies of the invention may be used as therapeutics. Therapeutic antibodies are generally agonists or antagonists, in that they either enhance or reduce, respectively, at least one of the biological activities of an beta secretase polypeptide.
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25 Antagonist antibodies of the invention are antibodies or binding fragments thereof which are capable of specifically binding to an beta secretase polypeptide, fragment, variant and/or derivative, and which are capable of inhibiting or eliminating the functional
40 activity of an beta secretase polypeptide *in vivo* or *in vitro*. In preferred embodiments, an antagonist antibody will inhibit the functional activity of an beta secretase polypeptide at least about 50 percent, and preferably at least about 80 percent. Agonist and
45 30 antagonist anti-beta secretase antibodies are identified by screening assays described below.
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Genetically Engineered Non-Human Mammals

Included within the scope of the present invention are non-human mammals such as mice, rats, rabbits, goats, or sheep in which one or both alleles of the gene encoding a native beta secretase polypeptide has (have) been disrupted ("knocked out") such that the level of expression of this gene is decreased or completely abolished. Such mammals may be prepared using techniques and methods such as those described in U.S. Patent No. 5,557,032. Such beta secretase knockout mammals have use in evaluating the effects of decreased beta secretase expression on Alzheimer's disease.

The present invention further includes non-human mammals such as mice, rats, rabbits, goats, or sheep in which the gene (or genes) encoding beta secretase polypeptides in which either the native form of the gene(s) for that mammal or a heterologous beta secretase polypeptide gene(s) is (are) over-expressed by the mammal, thereby creating a "transgenic" mammal. Such transgenic mammals may be prepared using well known methods such as those described in U.S. Patent No 5,489,743 and PCT Publication No. WO94/28122. Such transgenic mammals have use in evaluating the effects of over production of beta secretase on Alzheimer's disease.

The present invention further includes non-human mammals in which the promoter for one or more of the beta secretase polypeptides of the present invention is either activated or inactivated (using homologous recombination methods as described below) to alter the level of expression of one or more of the native beta secretase polypeptides. These mammals have uses similar to those set forth for transgenic and knockout mammals.

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- 54 -

10 Modulators of Beta Secretase Polypeptide Activity

In some situations, it may be desirable to identify molecules that are modulators, i.e., agonists or antagonists, of the activity of beta secretase polypeptide.

Natural or synthetic molecules that modulate beta secretase can be identified using one or more of the screening assays described below. Such molecules may be administered either in an *ex vivo* manner, or in an *in vivo* manner by local or IV injection, or by oral delivery, implantation device, or the like.

The following definition is used herein for describing the assays:

25 15 "Test molecule(s)" refers to the molecule(s) that is/are under evaluation for the ability to modulate beta secretase polypeptide activity. Such test molecule may be an agonist or antagonist of beta secretase.

30 20 Methods for identifying compounds which modulate beta secretase activity are encompassed by the present invention. In general, a beta secretase polypeptide can be incubated with a test molecule under conditions which permit the interaction of the test 35 25 molecule with beta secretase polypeptide, and the extent of the interaction can then be measured. The test molecule may be screened in a substantially purified form or in a crude mixture. Test molecules may be nucleic acid molecules, proteins, peptides, 40 30 carbohydrates, lipids or small molecular weight organic or inorganic compounds. Once a set of test molecules has been identified as binding to an beta secretase 45 35 polypeptide, the molecules may be further evaluated for their ability to increase or decrease beta secretase 50 35 activity.

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- 55 -

Measurement of the interaction of test
molecules with beta secretase polypeptides may be
carried out in several formats, including, without
limitation, enzymatic assays, cell-based assays,
solution-phase assays, immunoassays and *in vivo* assays.
In general, test molecules are incubated with an beta
secretase polypeptide for a specified period of time
and the extent of beta secretase activity can then be
determined by the biological activity assay set forth
herein, or by other appropriate assays such as
immunoassays.

The beta secretase agonist or antagonist may
be a protein, peptide, carbohydrate, lipid or small
molecular weight molecule which interacts with beta
secretase to regulate its activity. Potential protein
antagonists of beta secretase include antibodies which
bind to active regions of the polypeptide and inhibit
or eliminate at least once activity of beta secretase.
Molecules which regulate beta secretase polypeptide
expression may include nucleic acids which are
complementary to nucleic acids encoding an beta
secretase polypeptide, or are complementary to nucleic
acids sequences which direct or control expression of
beta secretase polypeptide, and which act as anti-sense
regulators of expression.

In some cases, it may be desirable to
evaluate two or more test compounds together for their
ability to modulate beta secretase polypeptide
activity. In these cases, the assays can be readily
modified by adding such additional test compound(s)
either simultaneous with, or subsequent to, the first
test compound.

In vitro assays such as the biological
activity assay described herein may be used
advantageously to rapidly screen large numbers of
compounds for effects on the activity of beta

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- 56 -

10 secretase. The assays may be automated to screen compounds generated using phage display, synthetic peptide and chemical synthesis libraries.

15 Compounds which increase or decrease beta
5 secretase activity may be screened in cell culture using cells and cell lines expressing beta secretase. Cells and cell lines may be obtained from any mammal, but preferably will be from human or other primate, canine, or rodent sources.

10 20 Beta Secretase Compositions and Administration

25 Therapeutic compositions of beta secretase polypeptides are within the scope of the present invention. Such compositions may comprise a
15 therapeutically effective amount of a beta secretase polypeptide, fragment, variant, or derivative in admixture with a pharmaceutically acceptable agent such as a pharmaceutically acceptable carrier. The carrier material may be water for injection, preferably
30 20 supplemented with other materials common in solutions for administration to mammals. Typically, a beta secretase polypeptide therapeutic compound will be administered in the form of a composition comprising purified polypeptide, fragment, variant, or derivative
35 25 in conjunction with one or more physiologically acceptable agents. Neutral buffered saline or saline mixed with serum albumin are exemplary appropriate carriers. Preferably, the product is formulated as a lyophilizate using appropriate excipients (e.g.,
40 30 sucrose). Other standard pharmaceutically acceptable agents such as carriers, diluents, and excipients may be included as desired. Other exemplary compositions comprise Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which may further include
45 35 sorbitol or a suitable substitute therefor.

50 Beta secretase pharmaceutical compositions

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- 57 -

10 typically include a therapeutically or prophylactically effective amount of beta secretase protein in admixture with one or more pharmaceutically and physiologically acceptable formulation agents selected for suitability
15 5 with the mode of administration. Suitable formulation materials or pharmaceutically acceptable agents include, but are not limited to, antioxidants, preservatives, coloring, flavoring and diluting agents, emulsifying agents, suspending agents, solvents,
20 10 fillers, bulking agents, buffers, delivery vehicles, diluents, excipients and/or pharmaceutical adjuvants. For example, a suitable vehicle may be water for injection, physiological saline solution, or artificial cerebrospinal fluid, possibly supplemented with other
25 15 materials common in compositions for parenteral administration. Neutral buffered saline or saline mixed with serum albumin are further exemplary vehicles. The term "pharmaceutically acceptable carrier" or "physiologically acceptable carrier" as
30 20 used herein refers to a formulation agent(s) suitable for accomplishing or enhancing the delivery of the beta secretase protein as a pharmaceutical composition.
35 The primary solvent in a composition may be either aqueous or non-aqueous in nature. In addition,
40 25 the vehicle may contain other formulation materials for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the composition may contain additional formulation
45 30 materials for modifying or maintaining the rate of release of beta secretase protein, or for promoting the absorption or penetration of beta secretase protein.
50 35 The beta secretase polypeptide compositions can be administered parentally. Alternatively, the compositions may be administered intravenously or subcutaneously. When systemically administered, the

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- 58 -

therapeutic compositions for use in this invention may
10 be in the form of a pyrogen-free, parentally acceptable
aqueous solution. The preparation of such
pharmaceutically acceptable protein solutions, with due
5 regard to pH, isotonicity, stability and the like, is
within the skill of the art.

15 Therapeutic formulations of beta secretase
polypeptide compositions useful for practicing the
present invention may be prepared for storage by mixing
20 the selected composition having the desired degree of
purity with optional physiologically acceptable
carriers, excipients, or stabilizers (*Remington's
Pharmaceutical Sciences*, 18th Edition, A.R. Gennaro,
ed., Mack Publishing Company [1990]) in the form of a
25 lyophilized cake or an aqueous solution. Acceptable
carriers, excipients or stabilizers are nontoxic to
recipients and are preferably inert at the dosages and
concentrations employed, and include buffers such as
30 phosphate, citrate, or other organic acids;
35 antioxidants such as ascorbic acid; low molecular
weight polypeptides; proteins, such as serum albumin,
gelatin, or immunoglobulins; hydrophilic polymers such
as polyvinylpyrrolidone; amino acids such as glycine,
glutamine, asparagine, arginine or lysine;
40 monosaccharides, disaccharides, and other carbohydrates
including glucose, mannose, or dextrans; chelating
agents such as EDTA; sugar alcohols such as mannitol or
sorbitol; salt-forming counterions such as sodium;
45 and/or nonionic surfactants such as Tween, pluronics or
30 polyethylene glycol (PEG).

The optimal pharmaceutical formulation will
45 be determined by one skilled in the art depending upon
the intended route of administration, delivery format
and desired dosage. See for example, *Remington's
35 Pharmaceutical Sciences*, 18th Ed. (1990, Mack
50 Publishing Co., Easton, PA 18042 pages 1435-1712).

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- 59 -

10 Such compositions may influence the physical state, stability, rate of in vivo release, and rate of in vivo clearance of the present beta secretase protein.

15 An effective amount of an beta secretase 5 polypeptide composition to be employed therapeutically will depend, for example, upon the therapeutic objectives such as the indication for which the beta secretase polypeptide is being used, the route of administration, and the condition of the patient.

20 10 Accordingly, it may be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect.

25 A typical daily dosage may range from about 0.1 µg/kg to up to 100 mg/kg or more, depending on the factors 15 mentioned above. Typically, a clinician will

30 administer the composition until a dosage is reached 20 that achieves the desired effect. The composition may therefore be administered as a single dose, or as two or more doses (which may or may not contain the same 35 amount of beta secretase polypeptide) over time, or as a continuous infusion via implantation device or catheter.

35 As further studies are conducted, information 25 will emerge regarding appropriate dosage levels for treatment of various conditions in various patients, 40 and the ordinary skilled worker, considering the therapeutic context, the type of disorder under treatment, the age and general health of the recipient, 45 will be able to ascertain proper dosing.

50 30 The beta secretase polypeptide composition to 45 be used for in vivo parenteral administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes. Where the 55 35 composition is lyophilized, sterilization using these

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- 60 -

10 methods may be conducted either prior to, or following, lyophilization and reconstitution. The composition for parenteral administration ordinarily will be stored in lyophilized form or in solution.

15 5 Therapeutic compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

20 10 Effective administration forms, such as (1) slow-release formulations, (2) inhalant mists, or (3) orally active formulations are also envisioned. The beta secretase pharmaceutical composition also may be formulated for parenteral administration. Such

25 15 parenterally administered therapeutic compositions are typically in the form of a pyrogen-free, parenterally acceptable aqueous solution comprising beta secretase in a pharmaceutically acceptable vehicle. The beta secretase pharmaceutical compositions also may include particulate preparations of polymeric compounds such as

30 20 polylactic acid, polyglycolic acid, etc. or the introduction of beta secretase into liposomes.

35 Hyaluronic acid may also be used, and this may have the effect of promoting sustained duration in the circulation.

40 25 A particularly suitable vehicle for parenteral injection is sterile distilled water in which beta secretase is formulated as a sterile, isotonic solution, properly preserved. Yet another preparation may involve the formulation of beta

45 30 secretase with an agent, such as injectable microspheres, bio-erodible particles or beads, or liposomes, that provides for the controlled or sustained release of the protein product which may then be delivered as a depot injection. Other suitable

50 35 means for the introduction of beta secretase include implantable drug delivery devices which contain the

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- 61 -

beta secretase.

The preparations of the present invention may include other components, for example parenterally acceptable preservatives, tonicity agents, cosolvents,

wetting agents, complexing agents, buffering agents, antimicrobials, antioxidants and surfactants, as are well known in the art. For example, suitable tonicity enhancing agents include alkali metal halides (preferably sodium or potassium chloride), mannitol,

sorbitol and the like. Suitable preservatives include, but are not limited to, benzalkonium chloride, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid and the like. Hydrogen peroxide may also be used as preservative.

Suitable cosolvents are for example glycerin, propylene glycol and polyethylene glycol. Suitable complexing agents are for example caffeine, polyvinylpyrrolidone, beta-cyclodextrin or hydroxypropyl-beta-cyclodextrin.

Suitable surfactants or wetting agents include sorbitan esters, polysorbates such as polysorbate 80, tromethamine, lecithin, cholesterol, tyloxapal and the like. The buffers can be conventional buffers such as borate, citrate, phosphate, bicarbonate, or Tris-HCl.

The formulation components are present in concentration that are acceptable to the site of administration. For example, buffers are used to maintain the composition at physiological pH or at slightly lower pH, typically within a pH range of from about 5 to about 8.

A pharmaceutical composition may be formulated for inhalation. For example, beta secretase may be formulated as a dry powder for inhalation. Beta secretase inhalation solutions may also be formulated in a liquefied propellant for aerosol delivery. In yet another formulation, solutions may be nebulized.

It is also contemplated that certain

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- 62 -

formulations containing beta secretase may be
10 administered orally. Beta secretase which is
administered in this fashion may be formulated with or
without those carriers customarily used in the
15 compounding of solid dosage forms such as tablets and
capsules. For example, a capsule may be designed to
release the active portion of the formulation at the
point in the gastrointestinal tract when
bioavailability is maximized and pre-systemic
20 degradation is minimized. Additional agents may be
included to facilitate absorption of beta secretase.
Diluents, flavorings, low melting point waxes,
vegetable oils, lubricants, suspending agents, tablet
disintegrating agents, and binders may also be
25 employed.

Another preparation may involve an effective
30 quantity of beta secretase in a mixture with non-toxic
excipients which are suitable for the manufacture of
tablets. By dissolving the tablets in sterile water,
35 or other appropriate vehicle, solutions can be prepared
in unit dose form. Suitable excipients include, but
are not limited to, inert diluents, such as calcium
carbonate, sodium carbonate or bicarbonate, lactose, or
calcium phosphate; or binding agents, such as starch,
25 gelatin, or acacia; or lubricating agents such as
magnesium stearate, stearic acid, or talc.

Additional beta secretase formulations will
40 be evident to those skilled in the art, including
formulations involving beta secretase in combination
30 with one or more other therapeutic agents. Techniques
for formulating a variety of other sustained- or
controlled-delivery means, such as liposome carriers,
45 bio-erodible microparticles or porous beads and depot
injections, are also known to those skilled in the art.
35 See, for example, the Supersaxo et al. description of
controlled release porous polymeric microparticles for

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- 63 -

the delivery of pharmaceutical compositions
10 (International Publication No. WO 93/15722;
International Application No. PCT/US93/00829) the
disclosure of which is hereby incorporated by
5 reference.

15 Regardless of the manner of administration,
the specific dose may be calculated according to body
weight, body surface area or organ size. Further
refinement of the calculations necessary to determine
10 the appropriate dosage for treatment involving each of
the above mentioned formulations is routinely made by
those of ordinary skill in the art and is within the
ambit of tasks routinely performed by them.
20 Appropriate dosages may be ascertained through use of
25 appropriate dose-response data.

30 The route of administration of the
composition is in accord with known methods, e.g. oral,
injection or infusion by intravenous, intraperitoneal,
intracerebral (intraparenchymal),
20 intracerebroventricular, intramuscular, intraocular,
intraarterial, or intralesional routes, or by sustained
release systems or implantation device which may
optionally involve the use of a catheter. Where
35 desired, the compositions may be administered
continuously by infusion, bolus injection or by
implantation device.

40 Alternatively or additionally, the
composition may be administered locally via
implantation into the affected area of a membrane,
30 sponge, or other appropriate material on to which beta
secretase polypeptide has been absorbed or
45 encapsulated.

50 Where an implantation device is used, the
device may be implanted into any suitable tissue or
35 organ, and delivery of beta secretase polypeptide may
be directly through the device via bolus, or via

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- 64 -

continuous administration, or via catheter using
10 continuous infusion.

Beta secretase polypeptide may be
administered in a sustained release formulation or
15 preparation. Suitable examples of sustained-release
preparations include semipermeable polymer matrices in
the form of shaped articles, e.g. films, or
microcapsules. Sustained release matrices include
polyesters, hydrogels, polylactides (U.S. 3,773,919, EP
20 58,481), copolymers of L-glutamic acid and gamma ethyl-
L-glutamate (Sidman *et al.*, *Biopolymers*, 22: 547-556
[1983]), poly (2-hydroxyethyl-methacrylate) (Langer *et
al.*, *J. Biomed. Mater. Res.*, 15: 167-277 [1981] and
Langer, *Chem. Tech.*, 12: 98-105 [1982]), ethylene vinyl
25 acetate (Langer *et al.*, *supra*) or poly-D(-)-3-
hydroxybutyric acid (EP 133,988). Sustained-release
compositions also may include liposomes, which can be
prepared by any of several methods known in the art
(e.g., Eppstein *et al.*, *Proc. Natl. Acad. Sci. USA*, 82:
30 3688-3692 [1985]; EP 36,676; EP 88,046; EP 143,949).

The beta secretase polypeptides, fragments
thereof, variants, and derivatives, may be employed
35 alone, together, or in combination with other
pharmaceutical compositions. The beta secretase
25 polypeptides, fragments, variants, and derivatives may
be used in combination with other medicinal compounds
such as, for example, cytokines, growth factors,
antibiotics, anti-inflammatories, and/or
chemotherapeutic agents as is appropriate for the
40 indication being treated.

In some cases, it may be desirable to use
45 beta secretase polypeptide compositions in an *ex vivo*
manner. Here, cells, tissues, or organs that have been
removed from the patient are exposed to beta secretase
35 polypeptide compositions after which the cells, tissues

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- 65 -

and/or organs are subsequently implanted back into the patient.

In other cases, an beta secretase polypeptide may be delivered through implanting into patients certain cells that have been genetically engineered, using methods such as those described herein, to express and secrete the polypeptides, fragments, variants, or derivatives. Such cells may be animal or human cells, and may be derived from the patient's own tissue or from another source, either human or non-human. Optionally, the cells may be immortalized. However, in order to decrease the chance of an immunological response, it is preferred that the cells be encapsulated to avoid infiltration of surrounding tissues. The encapsulation materials are typically biocompatible, semi-permeable polymeric enclosures or membranes that allow release of the protein product(s) but prevent destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissues.

Methods used for membrane encapsulation of cells are familiar to the skilled artisan, and preparation of encapsulated cells and their implantation in patients may be accomplished without undue experimentation. See, e.g., U.S. Patent Nos. 4,892,538; 5,011,472; and 5,106,627. A system for encapsulating living cells is described in PCT WO 91/10425 (Aebischer et al.). The cells, with or without encapsulation, may be implanted into suitable body tissues or organs of the patient.

Gene Therapy and Homologous Recombination

Further included in the scope of the present invention is production of beta secretase polypeptide by homologous recombination, and production of beta secretase polypeptide using control elements introduced

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- 66 -

10 into cells containing beta secretase DNA. For example, homologous recombination methods may be used to modify a cell containing a transcriptionally active beta secretase gene to produce a cell which does not express 5 therapeutically efficacious amounts of beta secretase.

15 Homologous recombination is a technique originally developed for targeting genes to induce or correct mutations in transcriptionally active genes (see Kucherlapati, *Prog. in Nucl. Acid Res. and Mol.*

20 *Biol.*, 36:301, 1989). The basic technique was developed as a method for introducing specific mutations into specific regions of the mammalian genome (Thomas et al., *Cell*, 44:419-428, 1986; Thomas et al., *Cell*, 51:503-512, 1987; Doetschman et al., *Proc. Natl. Acad. Sci.*, 85:8583-8587, 1988) or to correct specific 25 mutations within defective genes (Doetschman et al., *Nature*, 330:576-578, 1987). Exemplary homologous recombination techniques are described in U.S. 5,272,071 (EP 91 90 3051, EP Publication No. 505 30 500; and International Publication No. WO 91/09955).

35 Through homologous recombination, the DNA sequence to be inserted into the genome can be directed to a specific region of the gene of interest by attaching it to targeting DNA. The targeting DNA is a 25 nucleic acid molecule that is complementary to a region of the genomic DNA. Small pieces of targeting DNA that are complementary to a specific region of the genome are put in contact with the parental strand during the 40 DNA replication process. It is a general property of 30 DNA that has been inserted into a cell to hybridize, and therefore, recombine with other pieces of endogenous DNA through shared homologous regions. If 45 this complementary strand is attached to an oligonucleotide that contains a mutation or a different 35 sequence or an additional nucleotide, it too is incorporated into the newly synthesized strand as a 50

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- 67 -

10 result of the recombination. As a result of the proofreading function, it is possible for the new DNA to serve as the template. Thus, this new DNA is incorporated into the genome.

15 5 Attached to these pieces of targeting DNA are regions of DNA which may interact with the expression of a beta secretase protein. For example, a promoter/enhancer element, a suppresser, or an exogenous transcription modulatory element is inserted
20 10 in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired beta secretase protein. The control element controls a portion of the DNA present in the host cell genome.
25 15 Thus, the expression of beta secretase protein may be achieved not by transfection of DNA that encodes the beta secretase gene itself, but rather by the use of targeting DNA (containing regions of homology with the endogenous gene of interest) coupled with DNA
30 20 regulatory segments that provide the endogenous gene sequence with recognizable signals for transcription of a beta secretase protein.

35 35 In an exemplary method, expression of a desired targeted gene in a cell (i.e., a desired
40 25 endogenous cellular gene) can be altered by introducing an exogenous DNA molecule into a preselected site of genomic DNA. The exogenous DNA molecule typically includes at least a regulatory sequence, an exon and a splice donor site. This exogenous DNA is introduced
45 30 into the genomic DNA in a location so as to produce a new transcription unit in which the regulatory sequence, the exon and the splice donor site present in the exogenous DNA are operatively linked to the endogenous gene. As a result of introduction of the
50 35 exogenous DNA into the genomic DNA, the expression of the desired endogenous gene is altered.

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- 68 -

Altered gene expression, as used herein, encompasses activating (or causing to be expressed) a gene which is normally silent (unexpressed) in the cell as obtained, increasing expression of a gene which is not expressed at physiologically significant levels in the cell as obtained, changing the pattern of regulation or induction such that it is different than occurs in the cell as obtained, and reducing (including eliminating) expression of a gene which is expressed in the cell as obtained.

The present invention further relates to DNA constructs useful in the method of altering expression of a target gene. Exemplary DNA constructs typically comprise the following components: (a) a targeting sequence; (b) a regulatory sequence; (c) an exon; and (d) an unpaired splice-donor site. The targeting sequence in the DNA construct directs the integration of elements (a) - (d) into a target gene in a cell such that the elements (b) - (d) are operatively linked to sequences of the endogenous target gene. In another embodiment, the DNA constructs comprise: (a) a targeting sequence, (b) a regulatory sequence, (c) an exon, (d) a splice-donor site, (e) an intron, and (f) a splice-acceptor site, wherein the targeting sequence directs the integration of elements (a) - (f) such that the elements of (b) - (f) are operatively linked to the endogenous gene. The targeting sequence is homologous to the preselected site in the cellular chromosomal DNA with which homologous recombination is to occur. In the construct, the exon is generally 3' of the regulatory sequence and the splice-donor site is 3' of the exon.

If the sequence of a particular gene is known, such as the nucleic acid sequence of beta secretase presented herein, a piece of DNA that is complementary to a selected region of the gene can be

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- 69 -

synthesized or otherwise obtained, such as by
10 appropriate restriction of the native DNA at specific
recognition sites bounding the region of interest.
This piece serves as a targeting sequence upon
15 insertion into the cell and will hybridize to its
homologous region within the genome. If this
hybridization occurs during DNA replication, this piece
of DNA, and any additional sequence attached thereto,
will act as an Okazaki fragment and will be
20 backstitched into the newly synthesized daughter strand
of DNA. The present invention, therefore, includes
nucleotides encoding a beta secretase molecule, which
nucleotides may be used as targeting sequences.

Cell based therapy via genetic manipulation
25 is also included in the scope of the present invention.
In those situations where it is desirable to increase
the level of beta secretase activity in cells, beta
secretase cell therapy, e.g., implantation of cells
30 producing beta secretase, may be appropriate. This
embodiment encompasses implanting into patients cells
20 capable of synthesizing and secreting a biologically
active form of beta secretase. Such beta secretase-
producing cells may be cells that are natural producers
35 of beta secretase or may be recombinant cells whose
ability to produce beta secretase has been augmented by
25 transformation with a gene encoding the desired beta
secretase molecule or with a gene augmenting the
expression of beta secretase. Such a modification may
40 be accomplished by means of a vector suitable for
30 delivering the gene as well as promoting its expression
and secretion. In order to minimize a potential
45 immunological reaction in patients being administered a
beta secretase protein or polypeptide of a foreign
species, it is preferred that the natural cells
35 producing beta secretase be of human origin and produce
50 human beta secretase. Likewise, it is preferred that

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- 70 -

10 the recombinant cells producing beta secretase be transformed with an expression vector containing a gene encoding a human beta secretase molecule.

15 Implanted cells may be encapsulated to avoid infiltration of surrounding tissue. Human or non-human animal cells may be implanted in patients in biocompatible, semipermeable polymeric enclosures or membranes that allow release of beta secretase, but that prevent destruction of the cells by the patient's immune system or by other detrimental factors from the surrounding tissue. Alternatively, the patient's own cells, transformed to produce beta secretase ex vivo, could be implanted directly into the patient without such encapsulation.

20 15 Techniques for the encapsulation of living cells are known in the art, and the preparation of the encapsulated cells and their implantation in patients may be accomplished without undue experimentation. For example, Baetge et al. (International Publication No.

30 20 WO 95/05452) describe membrane capsules containing genetically engineered cells for the effective delivery of biologically active molecules. The capsules are biocompatible and are easily retrievable. The capsules encapsulate cells transfected with recombinant DNA

35 25 molecules comprising DNA sequences coding for biologically active molecules operatively linked to promoters that are not subject to down regulation in vivo upon implantation into a mammalian host. The devices provide for the delivery of the molecules from

40 30 living cells to specific sites within a recipient. In addition, see U.S. Patent Numbers 4,892,538, 5,011,472, and 5,106,627. A system for encapsulating living cells is described in PCT Application WO 91/10425 of Aebischer et al. See also PCT Application WO 91/10470

45 35 of Aebischer et al.; Winn et al., *Exper. Neurol.*, 113:322-329, 1991; and Aebischer et al., *Exper.*

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- 71 -

Neurol., 111:269-275, 1991.

10 In vivo and in vitro gene therapy delivery of
beta secretase is also envisioned. In vivo gene
therapy may be accomplished by introducing the gene
5 encoding beta secretase into cells via local injection
of a polynucleotide molecule or other appropriate
15 delivery vectors. (Hefti, J. Neurobiology., 25:1418-
1435, 1994). For example, a polynucleotide molecule
encoding beta secretase may be contained in an adeno-
20 associated virus vector for delivery to the targeted
cells (e.g., Johnson, International Publication No. WO
95/34670; International Application No.
25 PCT/US95/07178). The recombinant adeno-associated
virus (AAV) genome typically contains AAV inverted
20 terminal repeats flanking a DNA sequence encoding beta
secretase operably linked to functional promoter and
polyadenylation sequences.

30 Alternative viral vectors include, but are
not limited to, retrovirus, adenovirus, herpes simplex
20 virus and papilloma virus vectors. U.S. 5,672,344
(issued September 30, 1997, Kelley et al., University
of Michigan) describes an in vivo viral-mediated gene
35 transfer system involving a recombinant neurotrophic
HSV-1 vector. U.S. 5,399,346 (issued March 21, 1995,
25 Anderson et al., Department of Health and human
Services) provides examples of a process for providing
a patient with a therapeutic protein by the delivery of
40 human cells which have been treated in vitro to insert
a DNA segment encoding a therapeutic protein.
30 Additional methods and materials for the practice of
gene therapy techniques are described in U.S. 5,631,236
45 (issued May 20, 1997, Woo et al., Baylor College of
Medicine) involving adenoviral vectors; U.S. 5,672,510
(issued September 30, 1997, Eglitis et al., Genetic
35 Therapy, Inc.) involving retroviral vectors; and U.S.
50 5,635,399 (issued June 3, 1997, Kriegler et al., Chiron

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- 72 -

10 Ccrporation) involving retroviral vectors expressing cytokines.

15 Nonviral delivery methods include liposome-mediated transfer, naked DNA delivery (direct

5 injection), receptor-mediated transfer (ligand-DNA complex), electroporation, calcium phosphate precipitation and microparticle bombardment (e.g., gene gun). Gene therapy materials and methods may also include inducible promoters, tissue-specific enhancer-

10 promoters, DNA sequences designed for site-specific integration, DNA sequences capable of providing a selective advantage over the parent cell, labels to identify transformed cells, negative selection systems and expression control systems (safety measures), cell-

15 specific binding agents (for cell targeting), cell-specific internalization factors, transcription factors to enhance expression by a vector as well as methods of vector manufacture. Such additional methods and materials for the practice of gene therapy techniques

20 are described in U.S. 4,970,154 (issued November 13, 1990, D.C. Chang, Baylor College of Medicine)

25 electroporation techniques; WO 9640958 (published 961219, Smith et al., Baylor College of Medicine)

30 nuclear ligands; U.S. 5,679,559 (issued October 21,

35 1997, Kim et al., University of Utah Research Foundation) concerning a lipoprotein-containing system

40 for gene delivery; U.S. 5,676,954 (issued October 14, 1997, K.L. Brigham, Vanderbilt University involving

45 liposome carriers; U.S. 5,593,875 (issued January 14, 1997, Wurm et al., Genentech, Inc.) concerning methods

50 for calcium phosphate transfection; and U.S. 4,945,050 (issued July 31, 1990, Sanford et al., Cornell Research Foundation) wherein biologically active particles are

55 propelled at cells at a speed whereby the particles penetrate the surface of the cells and become incorporated into the interior of the cells.

10 Expression control techniques include chemical induced regulation (e.g., WO 9641865 and WO 9731899), the use of a progesterone antagonist in a modified steroid hormone receptor system (e.g., U.S. 5,364,791),
15 5 ecdisone control systems (e.g., WO 9637609), and positive tetracycline-controllable transactivators (e.g., U.S. 5,589,362; U.S. 5,650,298; and U.S. 5,654,168).

It is also contemplated that beta secretase
20 10 gene therapy or cell therapy can further include the delivery of a second prolypeptide. For example, the host cell may be modified to express and release both beta secretase and a second polypeptide of interest. Alternatively, the beta secretase and the second
25 15 polypeptide of interest may be expressed in and released from separate cells. Such cells may be separately introduced into the patient or the cells may be contained in a single implantable device, such as the encapsulating membrane described above.

30 20 Gene therapy can be used to decrease beta secretase polypeptide expression by modifying the nucleotide sequence of the endogenous promoter(s). Such modification is typically accomplished via homologous recombination methods. For example, a DNA
35 25 molecule containing all or a portion of the promoter of the beta secretase gene(s) selected for inactivation can be engineered to remove and/or replace pieces of the promoter that regulate transcription. Here, the TATA box and/or the binding site of a transcriptional
40 30 activator of the promoter may be deleted using standard molecular biology techniques; such deletion can inhibit promoter activity thereby repressing transcription of the corresponding beta secretase gene. Deletion of the TATA box or transcription activator binding site in the
45 35 promoter may be accomplished by generating a DNA construct comprising all or the relevant portion of the

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- 74 -

- 10 beta secretase polypeptide promoter(s) (from the same or a related species as the beta secretase gene(s) to be regulated) in which one or more of the TATA box and/or transcriptional activator binding site
- 15 5 nucleotides are mutated via substitution, deletion and/or insertion of one or more nucleotides such that the TATA box and/or activator binding site has decreased activity or is rendered completely inactive.
- 20 This construct, which also will typically contain at least about 500 bases of DNA that correspond to the native (endogenous) 5' and 3' DNA sequences adjacent to the promoter segment that has been modified, may be introduced into the appropriate cells (either *ex vivo* or *in vivo*) either directly or via a viral vector as
- 25 15 described above. Typically, integration of the construct into the genomic DNA of the cells will be via homologous recombination, where the 5' and 3' DNA sequences in the promoter construct can serve to help integrate the modified promoter region via
- 30 20 hybridization to the endogenous chromosomal DNA.
- 35 Other gene therapy methods may also be employed where it is desirable to inhibit the activity of one or more beta secretase polypeptides. For example, antisense DNA or RNA molecules, which have a
- 40 25 sequence that is complementary to at least a portion of the selected beta secretase polypeptide gene(s) can be introduced into the cell. Typically, each such antisense molecule will be complementary to the start site (5' end) of each selected beta secretase gene.
- 45 30 When the antisense molecule then hybridizes to the corresponding beta secretase mRNA, translation of this mRNA is prevented.
- 50 35 Alternatively, gene therapy may be employed to create a dominant-negative inhibitor of one or more beta secretase polypeptides. In this situation, the DNA encoding a mutant full length or truncated

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- 75 -

10 polypeptide of each selected beta secretase polypeptide can be prepared and introduced into the cells of a patient using either viral or non-viral methods as described above. Each such mutant is typically
5 designed to compete with endogenous polypeptide in its biological role.

15

Utility

10 Nucleic acid molecules of the invention may
20 be used to map the locations of the beta secretase gene and related genes on chromosomes. Mapping may be done by techniques known in the art, such as PCR amplification and *in situ* hybridization.

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In addition, the nucleic acid molecules can
15 be used as anti-sense inhibitors of beta secretase expression. Such inhibition may be effected by nucleic acid molecules which are complementary to and hybridize to expression control sequences (triple helix formation) or to beta secretase mRNA. Anti-sense
30 probes may be designed by available techniques using the sequence of beta secretase disclosed herein. Anti-sense inhibitors provide information relating to the decrease or absence of an beta secretase polypeptide in a cell or organism.

35

35 25 Hybridization probes may be prepared using the beta secretase nucleic acid molecules provided herein to screen cDNA, genomic or synthetic DNA libraries for related sequences. Regions of the DNA and/or amino acid sequence of beta secretase that
40 30 exhibit significant identity to known sequences are readily determined using sequence alignment algorithms disclosed above and those regions may be used to design
45 probes for screening.

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Further, the nucleic acid molecules of the
50 35 invention may be used for gene therapy. Nucleic acid molecules which express beta secretase *in vivo* provide

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- 76 -

information relating to the effects of the polypeptide
in cells or organisms.

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Beta secretase nucleic acid molecules,
fragments, and/or derivatives that do not themselves
5 encode biologically active polypeptides may be useful
as hybridization probes in diagnostic assays to test,
either qualitatively or quantitatively, for the
presence of beta secretase DNA or corresponding RNA in
mammalian tissue or bodily fluid samples.

15

10 Beta secretase polypeptide fragments,
variants, and/or derivatives, whether biologically
active or not, are useful for preparing antibodies that
bind to a beta secretase polypeptide. The antibodies
may be used for *in vivo* and *in vitro* diagnostic
20 purposes, such as in labeled form to detect the
presence of beta secretase polypeptide in a body fluid
or cell sample. Diagnosis of conditions such as
Alzheimer's disease, Down's syndrome, and amyloid
25 angiopathy may be accomplished by measuring beta-
secretase levels and activity from bodily tissue or
cell samples such as plasma, and comparing this data
against known "normal" standards for each such
30 condition. Beta-secretase levels can be measured by
ELISA or Western blot for example; beta-secretase
35 activity can be measured by the assays set forth in the
Examples below. Abnormal results of one or both assays
could indicate that a person is at high risk of
developing such diseases, and early treatment could be
40 started via administration of a beta-secretase
30 inhibitor, for example.

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Based on Northern blot data presented in the
Examples below, beta-secretase expression is relatively
high in the pancreas. This suggests that beta-
secretase may be implicated in diseases such as acute
45 and/or chronic pancreatitis, pancreatic cancer, and
50 pancreobiliary duct obstruction, and modulation of the

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- 77 -

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activity of beta-secretase may decrease the extent of, or completely prevent, such disorders.

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The beta-secretase antibodies may bind to a beta-secretase polypeptide so as to diminish or block at least one activity characteristic of beta secretase polypeptide, or may bind to a beta-secretase polypeptide to increase its activity.

20

A plasmid containing cDNA encoding full length beta secretase has been transformed into in *E. coli* cells, and the cells have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 on March 11, 1999. The cells have been designated as accession no. 207159.

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15 A plasmid containing cDNA encoding amino acids 1-460 of beta-secretase fused to the Fc portion of human IgG beta has been transformed into in *E. coli* cells, and the cells have been deposited with the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209 on March 11, 1999. The cells have been designated as accession no. 207158.

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35 The following examples are intended for illustration purposes only, and should not be construed 25 as limiting the scope of the invention in any way.

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- 78 -

Example I: Production of an APP Expressing Cell Line

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The human APP-695 cDNA containing the Swedish mutation (referred to as "APP Δ NL" the sequence of which
15 has been published Mullan et al., *Nature Genetics* 1:
345-347 [1992]) was digested with the restriction enzymes *Hind*III and *Not*I, and the approximately 2.6 kb
20 *Hind*III - *Not* fragment was inserted into the vector pCMVi (Cell & Molecular Technologies Inc; Lavallette,
25 NJ) to generate the vector pCMVi-APP Δ NL).

25

Human embryonic kidney 293 cells containing the SV40 large T antigen (Cell & Molecular Technologies, Lavallette, NJ) were maintained in high glucose Dulbecco's modified Eagle's medium supplemented
30 with about 10 percent fetal calf serum. At approximately 70 percent confluence the cells were co-transfected with pCMVi-APP Δ NL and pRSV-Puro (Cell &
35 Molecular Technologies Inc; Lavallette, NJ) using the standard calcium phosphate method (Gorman et al, *DNA Prot. Eng. Tech.* 2: 3-10 [1990]). After about forty eight hours, puromycin resistant colonies were selected by adding puromycin (Sigma Inc., St. Louis, MO) at about 5 micrograms/ml to the culture medium.
40 Fourteen days later, individual resistant colonies were isolated, using a pipetteman P200 (Rainin, Emeryville, CA) expanded into a 24 well dish and allowed to grow
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10 confluent. The conditioned media were analyzed for the presence of secreted APP by Western dot blot using the monoclonal antibody 22C11 (Boehringer Manheim Corp., Indianapolis, IN).

15 5 One colony was grown up and a cell line referred to as "293T-APPsw101" was generated from it.

20 Example II: Construction of a Human Embryonic Kidney Cell Expression Library

10 10 Approximately 10^3 293 human embryonic kidney cells (ATCC CRL-1573), which are known to express the beta secretase gene, were grown to about 80 percent confluence in DMEM high glucose media supplemented with
25 15 about 10 percent fetal bovine serum using T225 tissue culture flasks (Corning/Costar, Encinitas, CA). Cells were harvested in about 3 ml of 0.5 mM EDTA in PBS and
30 35 pelleted at about 1100 RPM in a Beckman table top centrifuge. The cells were washed twice with cold PBS
35 40 and snap frozen in liquid nitrogen as a dry cell pellet. Poly A+ mRNA was harvested from the cell pellet using the FastTrack™ method of mRNA isolation (Invitrogen, San Diego CA) according to the
40 45 manufacturer's protocol. An oligo dT primed,
45 50 directional cDNA expression library was prepared from about three µg of this poly A+ mRNA using the Superscript™ plasmid system for cDNA synthesis (Gibco

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- 80 -

10 BRL, Gaithersburg, MD) according to the manufacturer's protocol. *Sai I* adaptors were ligated to the double stranded cDNA following the manufacturer's instructions.

15 5 After adaptor ligation, the cDNA was digested to completion with *Not I*, and size fractionated as per the manufacturer's protocol by column chromatography over a Sephadryl™ S-500 column which excludes cDNAs of 20 500 bp and smaller (provided by the manufacturer).

25 10 Column fractions were counted on a Beckman scintillation counter and Cherenkov counts were obtained for each fraction. cDNAs from fractions 10-12 were ethanol precipitated and resuspended in about 22 30 30 microliters of nuclease free water. The cDNA products 35 15 which averaged about 2.0 kb in length were directionally ligated into a CMV based expression vector (comparable to pRK-5; Pharmingen Inc., San Diego, CA) which had been previously digested with *NotI* and *SaiI*. The ligated cDNA was then introduced into

40 20 electrocompetent ElectroMax™ DH10B *E.coli* cells (Gibco BRL, Gaithersburg, MD) using standard electroporation procedures. The cDNA library was titered by serial dilution of the transformation cell mixture.

45 45 The cDNA library was subdivided into pools of 50 25 100, plated on LB agar plates containing about 100 micrograms/ml Ampicillin, and allowed to grow for about twenty hours. Cell colonies from each independent

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- 81 -

plate were then harvested in about two ml of 2xYT
10 media, transferred to deep-well 96 well dishes and
allowed to grow for an additional five to six hours at
37° C. The cells were pelleted by centrifugation at
15 3000 RPM in a Beckman table top centrifuge and DNA was
prepared using the TurboPrep™ method of DNA isolation
(Qiagen Inc., Chatsworth, CA) according to the
manufacturer's protocol. DNA from each pool was
20 arrayed in 96 well Corning Costar UV plates (CORNING
10 Costar, Charlotte, NC) and quantitated by O.D. 260
using a Molecular Devices Spectra Max plus (Molecular
25 Dynamics, Sunnyvale, CA).

A duplicate of each master cDNA 96 well plate
30 was prepared. Each well of the duplicate plate
15 contained about 75 microliters of Optimem I medium
(Life Technologies Inc, Gaithersburg, MD) and about 300
ng of cDNA library. About 75 microliters of a mixture
35 of 12 microliters DMRIE-C Lipid per ml of Optimem I
medium (Life Technologies Inc., Gaithersburg, MD) was
20 added to each well of the 96 well plate using a
40 Multipette (Sagian Inc. Indianapolis, IN) robotic
pipettor. The DMRIE C was allowed to complex for about
30 minutes. Meanwhile, the media was removed from a 96
45 well dish in which each well had been plated with about
25 15,000 cells/well of the 293T-APPsw 101 cell line two
days before. About 125 microliters of the DNA/Lipid
50 complex was added to each well of cells. The cells

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- 82 -

10 containing the DNA/lipid mixture were then incubated at
37 degrees C, 5 percent CO₂ for about five hours.

15 After incubation, 125 µl Optimem I supplemented with 20
percent FBS and 2x Penicillin/Streptomycin (0.1 unit/ml
5 Penicillin, 0.1 µg/ml Streptomycin) (Life Technologies
Inc., Gaithersburg, MD) was added to stop the
transfection. The following morning, the media was
20 removed and a fresh medium containing high glucose
DMEM, 10 percent FBS, and Pen/Strep was added, and the
10 cells were allowed to recover for about 24 hours.

25 Approximately 48 hours post transfection, fresh media
was added and the cells were incubated for 6-8 hours.
The medium from each well was then harvested, diluted
30 1:1 in Superblock (Pierce Inc., Rockford, IL), stamped
15 into pre-coated ELISA plates and analyzed for the
presence of A-beta as described below.

35 Example III: Beta-Secretase Activity Assays With
Cultured Cells

40 Beta secretase generates two forms of the
A-beta peptide, a 40 amino acid form and a 42 amino
acid form. In most people, about ninety percent of the
45 A-beta is the 40 amino acid form, and about 10 percent
25 is the 42 amino acid form. However, beta-amyloid
plaques consist primarily of the 42 amino acid form,

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- 83 -

10 and in those individuals with familial Alzheimer's disease, there is a larger proportion of the 42 amino acid form as compared with the 40 amino acid form.

15 To analyze the level of A-beta in the cell culture medium obtained from each well of each plate,
5 the following two assays were conducted on each medium.

20 A. Assay for Total Beta Amyloid Peptide

A monoclonal antibody against amino acids
10 17-24 of A-beta (Senetek Inc., St Louis, MO, item number 220-10) referred to as m4G8, was diluted to a
25 concentration of about 150 micrograms/ml in coating buffer (1.59g/l Na₂CO₃, 2.93g/l NaHCO₃, pH 9.69). About ninety microliters per well of coating buffer was then
30 dispensed on to a polystyrene high binding 96 well flat-bottomed plate (Corning Costar, New York, NY; item number 3590). About ten microliters per well of the
35 m4G8 antibody diluted to about 150 micrograms/ml were added to the plate coating resulting in a final
40 concentration of about 1.5 micrograms per well of m4G8. Each plate is then incubated overnight at 4C on a plate mixer. The plates were then washed with wash buffer
45 (7.1108g/l Tris-HCl: C₆H₁₁NO₂-HCl, 0.5984g/l Tris Base: C₆H₁₁NO₂, 8.766g NaCl, 0.5ml/l polyoxyethylene sorbitan
50 monolaurate: C₁₂H₂₅CO₂) four times, and the non-specific binding sites were blocked with 300 microliters per well of Superblock TBS (Pierce Chemical Company,

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- 84 -

10 Rockford, IL 61105 item number 375352Z) for about three hours at room temperature.

15 The samples used for calibration were prepared from a commercially obtained solution of Ab₁₋₄₀,
5 (5 micrograms/ml in DMSO; Quality Control Biochemicals,
Inc, MA). The most concentrated sample was a
20 40,000 pg/ml solution, and was prepared by dilution into Superblock TBS (Pierce Chemical CO., Rockford, IL 61105). Sequential dilutions were then made in diluent
10 to obtain 20,000, 10,000, 5,000, 2,500, 1,250, 625,
25 312.5, 156.25, 78.125 and 39.06 pg/ml.

30 One hundred microliters per well calibrators or 2.5 microliters per well of samples were applied to each well of each the microtiter plate; to each well
35 15 was previously added one hundred microliters Superblock TBS plus m4G8 antibody. The plates were covered and incubated at about 4C overnight with mixing after which they were washed four times with wash buffer at about
4C.

40 To assess the amount of A-beta in each sample, the antibody m6E10-biotin raised against residues 1-17 of A-beta (Senetek Inc., St Louis, MO, item number 340-10), was diluted to a final
45 concentration of about 0.5 micrograms/ml into antibody
50 25 diluent (Superblock TBS as described above) containing 2 percent normal mouse serum (Sigma Chemical Co., St. Louis, MO; item number S-3509) and 2 percent normal

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- 85 -

10 goat serum (Gibco/BRL, Grand Island, NY; item number
15210-064). About 100 microliters of this diluted
antibody were added to each well. The plate was
covered and incubated for about 2 hours at 4C with
15 mixing. The plate was then washed four times with wash
buffer at 4C.

20 Europium labeled streptavidin (Wallac;
Gaithersburg, MD; item number 1244-360) was diluted
1:1000 in Assay Buffer (Wallac; item number 1244-106)
10 to a final concentration of about 100 ng/ml. About one
hundred microliters of this diluted streptavidin were
25 then added to each well. Each plate was covered and
incubated for about 1 hour at 4C with mixing. Each
plate was washed four times with wash buffer at 4C
30 after which about 100 microliters of Enhancement
Solution (Wallac; item number 1244-105) was added to
each well. The plate was then mixed for at least 5
35 minutes at room temperature.

20 Delfia time resolved fluorescence was read
40 with a Wallac Victor 1420 multilabel counter using an
excitation wavelength of 340 nm and an emission
wavelength of 613 nm.

45 B. Assay for the 42 Amino Acid Form of A-Beta

25 A rabbit polyclonal antibody which
specifically recognizes residue 42 of A-beta (Quality
50 Controlled Biochemicals, MA 01748-2215; item number

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- 86 -

44344) was diluted to about 2.5 micrograms/ml in the
10 diluent Superblock TBS (Pierce Chemical Co. Rockford,
IL 61105) and was added to each Reacti-Bind goat-anti-
rabbit IgG-Fc specific plate (Pierce Chemical Co.;
15 Rockford, IL 61105 item number NC1513). The plates
were then incubated for about one hour at room
temperature on a plate mixer with vigorous mixing.
20 Following incubation, the plates were washed with wash
buffer TBS-Tween (0.5 percent) four times, and the non-
10 specific binding sites were blocked with about 100
microliters per well of Superblock TBS.
25

The standard curve samples were prepared from
a stock solution of A-beta-1-42 peptide in DMSO at a
concentration of 5 micrograms/ml. The highest standard
30 15 was prepared at a final concentration of 40,000 pg/ml
in Superblock TBS. Sequential dilutions were made
using Superblock TBS to obtain final concentrations of
35 20,000, 10,000, 5,000, 2,500, 1,250, 625, 312.5,
156.25, 78.125 and 39.06 pg/ml.

40 20 About one hundred microliters per well of
each standard, or 17.5 microliters per well of each
sample was applied to each well of the microtiter
plates to which about 100 microliters of Superblock TBS
45 had been previously added. The plates were covered and
25 incubated at about 4C overnight with mixing. The
plates were then washed four times with wash buffer
50 containing about 0.5 percent TBS-Tween-20™ at 4C.

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- 87 -

10 Detection antibody, m4G8-biotin (Senetek; St.
Louis, MO; item number 240-10) was diluted to about 0.5
micrograms per ml in a solution containing Superblock
TBS, 2 percent normal mouse serum, 2 percent normal
15 goat serum, and about 100 microliters of the diluted
antibody were added to each well. Each plate was
covered and incubated for about 2 hours at 4C with
mixing. Each plate was then washed four times with
20 standard wash buffer at 4C.

25 10 Europium labeled streptavidin (Wallac,
Gaithersburg, MD; item number 1244-360) was diluted
1:1000 in Assay Buffer (Wallac, Gaithersburg, MD; item
number 1244-106) to a final concentration of about 100
ng/ml. About one hundred μ l per well were then added
30 15 to each well. The plates were covered and incubated
for about 1 hour at 4C with mixing. Each plate was
washed four times with wash buffer at 4C, after which
35 about 100 μ l of Enhancement Solution (Wallac,
Gaithersburg, MD; item number 1244-105) were added to
40 20 each well. Each plate was then mixed for at least 5
minutes at room temperature.

45 25 Delfia time resolved fluorescence was read
with a Wallac Victor 1420 multilabel counter using an
excitation wavelength of about 340 nm and an emission
25 wavelength of about 613 nm.

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- 88 -

Example IV: Isolation of a Beta-Secretase cDNA

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Approximately 864,000 independent clones were screened in the assays described above. Positive 15 clones were selected based on the following two criteria:

- 20 1) increased A-beta 42 and/or total A-beta levels by more than two standard deviation points as compared with the plate average; and
- 10 2) selective skewing of the A-beta 42/ total A-beta ratio towards A-beta 42 production.

25

Based on these criteria, about 144 putative positive pools of cDNAs were replated onto new master 30 plates for a second round of analysis. An aliquot of 15 the cDNA from each pool was transfected into 293T-APPsw101 (described above) using procedures described 35 above. Transfections were conducted in duplicate. Those pools of cDNAs which consistently met positive 20 clone criteria were subsequently replated and diluted 40 from pools of about 100 clones to pools of about 20 clones and then to single clones. Single clones were diluted 1:10 into pCMV β vector DNA since the assumption 45 was made that severe over-expression of a protease 25 might result in toxicity to the cells.

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One pool, termed A-11, exhibited a dramatic skewing of the A-beta 42/A-beta total ratio. In

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- 89 -

10 duplicate assays, this pool gave a ratio of about 0.8,
and 1.0, respectively, whereas the plate average ratio
for all plates ranged from about 0.5 to about 0.7.
15 When A-11 was subsequently re-plated, it showed a
modest yet consistent skewing of the A-beta 42/A-beta
total ratio. In addition, A-11 had a standard
deviation of greater than 2 for the increase in A-beta
20 42 levels over the plate average. The ratio skewing
seemed to be somewhat dependent upon the density of
10 cells at the time of transfection. When the cells were
less dense at time of transfection, the skewing
25 appeared to be more robust. As pool A-11 was broken
down, it exhibited enhanced signal strength by both of
the above criteria.

30 15 The results of the three assays for pool A-11
as well as the plate average are set forth below in
Table II as total Europium counts. The data are
35 presented as an average of duplicate transfections
where each transfection was run in duplicate assays.

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- 90 -

Table II

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| Assay | Pool A-11 | Plate Average |
|------------------------------------|-----------|---------------|
| Re-Arrayed Pool of 100 | | |
| A-beta 42 | 94,397 | 72,858 |
| Total | 177,356 | 164,334 |
| A-beta 42/Total | 0.53 | 0.44 |
| Pool of 20 | | |
| A-beta 42 | 178,212 | 133,402 |
| Total | 448,660 | 388,093 |
| A-beta 42/Total | 0.397 | 0.34 |
| Single Clone (β -Secretase) | | |
| A-beta 42 | 327,505 | 230,518 |
| Total | 295,438 | 363,141 |
| A-beta 42/Total | 1.1 | 0.63 |

When pool A-11 was diluted to single clones,
 5 several of these clones were sequenced using standard
 methods. All of the sequenced clones possessed an
 identical nucleotide sequence. After sequencing, one
 45 of these clones was inserted into the vector
 pCMV β -secretase. Further sequence analysis
 10 indicated that the sequence possessed many sequence

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- 91 -

similarities to the aspartic protease family, and
10 encoded a novel aspartic protease containing both a
putative signal sequence and a transmembrane domain.
The cDNA sequence of this aspartic protease, identified
15 5 as a novel sequence encoding beta-secretase, is set
forth in Figure 1A and 1B. The putative amino acid
sequence as translated from the cDNA is set forth in
Figure 4, and this sequence possesses certain motifs
20 characteristic of an aspartic proteases, such as two
10 putative active site domains. The propeptide consists
of amino acids 23-45. The signal peptide spans amino
25 acids 1-22, and the transmembrane domain spans amino
acids 461-477. The enzyme appears to be cleaved *in*
vivo between amino acids 22 and 23 in some cases to
30 15 remove the signal peptide, and in addition between
amino acids 45 and 46 to remove the propeptide in other
cases. The active site domains are believed to be
35 amino acids 93-96 and 289-292. Hence, it is believed
that the mature, active beta secretase enzyme spans
20 amino acids 46-501. Based on computerized three
dimensional structure analysis of human beta amino acid
sequence, it is expected that the fragments 62-420, 73-
420, 83-420, 90-420, 62-417, 73-417, 83-417, 90-417,
45 25 62-410, 73-410, 83-410, 90-410, 62-402, 73-402, 83-402,
and 90-402 would all be biologically active.

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- 92 -

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Example V: Identification of the Mouse and Rat
Orthologs of Beta-Secretase

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To identify the mouse and rat beta-secretase cDNAs, a FASTA search was performed using the full length human beta-secretase cDNA to search an Amgen database. For conducting this search, the scoring matrix used was GenRunData.fastaDNA.cmp, together with a constant pam factor. The Gap creation penalty and Gap extension penalty were set for 12.0 and 4.0, respectively. Four ESTs were identified, three for mouse and one for rat.

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The three mouse sequences, which were 351 base pairs, 411 base pairs, and 364 base pairs in length were found to overlap. Thus, a complete mouse beta-secretase cDNA was obtained. This mouse cDNA was about 1923 base pairs in length and included about 95 nucleotides of 5 prime untranslated sequence, about 1503 nucleotides of open reading frame encoding 501 amino acids, and approximately 325 nucleotides of 3 prime untranslated sequence.

The single rat clone obtained encoded full length beta-secretase and was about 2158 nucleotides in length and contained 427 nucleotides of 5 prime untranslated sequence and 225 nucleotides of 3 prime untranslated sequence. The rat beta secretase is also 501 amino acids in length.

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- 93 -

10 The full length mouse and rat beta-secretase cDNAs share approximately 93 percent and 91 percent identity, respectively, at the nucleic acid level with the human full length beta-secretase cDNA.

15 5 The mouse cDNA sequence containing only the full length coding region is set forth in Figure 2A and 2B (SEQ ID NO:2) and the corresponding amino acid sequence is set forth in Figure 5 (SEQ ID NO:5).

20 10 The rat cDNA sequence containing only the full length coding region is set forth in Figure 3A and 3B (SEQ ID NO:3) and the corresponding amino acid sequence is set forth in Figure 6 (SEQ ID NO:6).

30 35 15 Example VI: Northern Blot Analysis of Beta-Secretase Transcripts

35 Northern blot analysis was performed to identify those tissues in which the beta-secretase
40 20 transcript is present. A probe for use in Northern blot analysis was generated by digesting the human β -secretase cDNA with *Pst I* for about three hours at 37° C and running the restriction digest on an 0.8 percent agarose gel to separate the fragments. The
45 25 approximately 772 base pair ("bp") *Pst I* fragment extending from nucleotide 318 to nucleotide 1090 of the
50 50 cDNA was isolated and gel purified using the QiaQuick®

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- 94 -

gel purification system (Qiagen, Chatsworth, CA). The
10 isolated, gel pure fragment was quantitated by
estimation on a one percent agarose gel. About 25 ng
of this fragment was denatured by boiling for 5
15 minutes, and then quenching on ice for 2 minutes. The
fragment was then radioactively labeled with alpha 32P-
dCTP using the High Prime DNA labeling kit (Boehringer
Manheim, Indianapolis, IN) according to the
20 manufacturer's protocol. Human multiple tissue
northern blots were purchased (Clonetech, Palo Alto,
10 CA) and first prehybridized in Clontech Express™
25 hybridization buffer for about one hour at about 65°C.
Following prehybridization, the labeled probe was
denatured by boiling for about five minutes and
30 15 quenching on ice for 2 minutes, and then added to the
hybridization buffer containing the Northern blots.
The blots were allowed to hybridize for about two hours
35 at about 65°C. After hybridization the blots were
washed in 2xSSC for 30 minutes at room temperature,
20 followed by 3 successive washes in 0.2xSSC containing
40 0.1 percent SDS at about 60°C for 30 minutes. The
blots were dried briefly and exposed to autoradiography
film for 72 hours at about -80°C.

45 The results are shown in Figure 7. The lane
25 contents are described in the Brief Description of the
Figures. Three different RNA transcripts of
50 approximately. 7 kb, 4.4 kb and 2.6 kb are apparent.

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- 95 -

10 All are of low abundance and expression is detected in
most tissues. The highest levels are observed in
pancreas and brain.

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Example VII: Beta-Secretase Protein Detection

20 An antibody to the carboxy terminus of beta-
secretase was raised as follows. A synthetic peptide
10 with the sequence CLRQQHDDFADDISLLK (SEQ ID NO:7)
corresponding to amino acids 485-501 of beta-secretase
25 was generated using standard methods (see below). The
peptide was then conjugated to the carrier protein KLH
by adding 5 mg of peptide to Pierce maleimide
30 conjugation buffer (Pierce Chemical Co.; Rockford, IL;
item number 77164), after which 0.5 ml of Pierce
maleimide activated KLH (5 mg) (Pierce Chemical Co.;
35 item number 77105) was added. The solution was
incubated for about 2 hours at room temperature, and
20 then run through a D-Salt™ Dextran column (Pierce
Chemical Co.; item number C43233) to remove the EDTA.
A one ml fraction from the column was mixed 1:1 with
40 Titermax Research Adjuvant (CytRx Corp., Norcross,
Georgia; item number R-10), prepared as an oil and
45 water emulsion using 18 gauge double-hubbed needles
(Popper and Sons, New Hyde Park, N.Y) and loaded into 1
50 c.c. syringes fitted with 21 gauge needles. Three New

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10 Zealand White rabbits were each injected intra-muscularly (IM) at two injection sites (0.05 ml per site). Four and six weeks later, the rabbits were boosted IM, again at two sites, 0.05 ml per site. The
15 5 first sample bleed of 5 ml was drawn at 6 weeks. Two weeks later the second test bleed was drawn and tested for immunoprecipitation of β -secretase from transfected
20 cells. The results indicated that a beta-secretase specific antibody had been generated.

10 The antibody was coupled to Protein A Sepharose using standard procedures for use in
25 immunoprecipitation assays. The preimmune serum (negative control) was coupled in the same manner.
30 Coupling was conducted as follows:

15 Protein A Sepharose CL-4B beads (Amersham
Pharmacia Biotech, Inc, Piscataway, NJ; item number 17-
35 0780-01) were suspended at about 250 mg/ml in BSA/TBS
(50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2% BSA). About 1
ml of the resuspended beads was mixed with about 250 μ l
20 of antiserum. The mixture was incubated at room
temperature, rocking gently, for about one hour. The
beads were then washed with about 10 ml of borate
40 buffer (0.2 M sodium borate, pH 9.0). After about five
minutes of gentle rocking, the mixture was spun at
45 3000g for about five minutes and the supernatant was
discarded. This washing procedure was then repeated,
50 after which the beads were resuspended in about ten ml

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- 97 -

10 of borate buffer. Before adding the coupling agent,
about 100 µl of the slurry was removed for gel
electrophoresis analysis (tube A, see below).

15 The antiserum was then covalently coupled to
5 the beads by adding about 50 mg of solid dimethyl
pimelimidate (DMP, Pierce Chemical Co., Rockford, IL;
item number 21666). The final concentration of DMP was
20 about 20 mM and had a pH of at least 8.3. After
incubating the mixture at room temperature for about
10 thirty minutes with gentle rocking, a 100 microliter
25 aliquot was removed (tube B, see below); the remainder
of the mixture was spun at about 3000 x g for about 5
minutes.

30 The coupling reaction was terminated by
15 washing the beads in about 10 ml of 0.2M ethanalamine,
pH 8.0. After mixing, the solution was spun at about
35 3000 x g for about 5 minutes. The beads were
resuspended in about 10 ml of ethanalamine and
incubated for about 2 hours at room temperature with
20 gentle rocking. The beads were then spun at about 3000
x g for about 5 minutes, after which the pellet was
40 washed briefly with about 2 ml glycine (100 mM glycine,
pH 3.0), followed by spinning again at about . 3000 x g
45 for about 5 minutes.

50 25 The beads were rinsed with about 2 ml Tris
(100 mM, pH 8.0) and spun at about 3000 x g for about 5
minutes, after which the beads were resuspended in ..

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- 98 -

about 1 ml PBS containing 0.01 percent thimerosal.

10 The beads were stored at 4°C. An aliquot (about 20 µl) of this final product (tube C) was run on SDS-PAGE gel along with tubes A and B (see above) to check the
15 efficiency of coupling. The beads were prepared for SDS-PAGE by pelleting and resuspending them in about 30 µl of SDS sample buffer (Novex, San Diego, CA; item number LC2676), after which they were heated at about
20 85°C for about 10 minutes and then run on a 10 percent
10 Tris Glycine gel (Novex, San Diego, CA) at 100V for
25 about 2-3 hours. The gel was and stained with Coomassie blue.

30 To evaluate the presence of beta-secretase in human brain tissue, human Alzheimer's disease and age-matched control brains were obtained from Sun Health Research Institute (Sun City, Arizona) and homogenized according to the following protocol:

35 Pieces of parietal cortex of approximately 1 gram wet weight were finely chopped while frozen, and
40 then placed in a Dounce glass homogenizer. Lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 percent NP-40, 12 mM CHAPS, 0.2 percent BSA, 2 mM EDTA, 20 mM PMSF, 10 µM leupeptin, 1 µM pepstatin A, 2 µg/ml aprotinin, 0.1 mM Pefabloc) was added in a proportion
45 of 2 ml buffer per 1 g brain tissue, and the mixture
50 was homogenized using 50 strokes with a tight fitting

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- 99 -

10 plunger. The homogenate was spun at 20,000 x g for
about 10 minutes at 4°C. The supernatant was used for
the immunoprecipitation and Western blotting as
described below. Remaining material was stored at
15 -80°C. The supernatant concentration was assumed to be
approximately 500 mg protein /ml based on the starting
wet weight.

20 The supernatants were immunoprecipitated as
described immediately below with either the antibody or
10 with preimmune serum coupled to protein A Sepharose
25 (prepared as described above), separated on SDS-PAGE,
and Western blotted with the pooled antisera. As a
positive control, lysates made from 239T-APPsw101 cells
30 described above transiently transfected with the full
15 length beta-secretase expression construct were
analyzed under the same conditions.

35 For each immunoprecipitation, about 50 µl of
brain supernatant prepared as described above (about 25
mg wet weight equivalent) or the lysate made from 101
20 cells transiently transfected with the beta-secretase
expression construct (about 1/20 of the volume of each
dish) were used. The brain supernatant and the lysate
40 were precleared in bulk (about 100 µl homogenate
containing about 25 µl Protein A Sepharose) by
45 incubating at about 4°C for 30 minutes with gentle
rocking. The tubes were centrifuged at about 960 x g
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for about 5 minutes. The supernatants (about 50 µl)
10 were immunoprecipitated with about 40 µl of the
Protein-A- beta-secretase antisera or Protein-A-
preimmune serum. The volume of the immunoprecipitation
15 5 was brought up to about 500 µl with TBS (50 mM Tris, pH
7.5, 150 mM NaCl) and the reaction was carried out at
about 4°C for about 3 hours with rocking. The beads
20 were pelleted at about 960 x g for about 5 minutes and
washed three times with STEN buffer (1x STEN = 50 mM
Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.2 percent
25 NP-40) for about 15 minutes at about 4°C with rocking.
The beads were pelleted after each wash at about 960 x
g for about 5 minutes. The first wash buffer was 0.5 M
30 STEN (1x STEN + 0.5 M sodium chloride, 5 µg/ml
leupeptin, 5 µg/ml aprotinin). The second wash buffer
35 was SDS-STEN (1x STEN + 0.1% SDS, 5 µg/ml leupeptin, 5
µg/ml aprotinin), and the third wash buffer was 1x
STEN. After washing, about 15 µl of SDS-Sample Buffer
40 (Novex, San Diego, CA; item number LC2676) was added
20 and the samples were stored at -20°C overnight.
The samples were heated at about 95°C for
45 about 5 minutes and loaded onto a 10-20 percent Tris-
Tricine gel (Novex San Diego, CA ; item number EC6625).
The gel was run at 100V for 2-3 hours and then
50 25 transferred to PVDF (Novex, San Diego, CA; item number

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- 101 -

10 LC2002) using the Bio Rad Trans Blot Cell at about 400 mA for about 2 hours at 4°C in a solution of 200 mM glycine, 25 mM Trizma base and 20 percent methanol.

15 The Western blot was then analyzed for the presence of beta secretase as follows. The Western blot was wetted in methanol for about 30 seconds then rinsed with TBST (10 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 0.05 percent Tween-20) before blocking with 5 percent dry milk in TBST for about one and one-half 20 hours at room temperature, with rocking. The Western blot was incubated with primary beta-secretase antibody (prepared as described above) that had been diluted about 1:1000 in PBS containing 1 percent BSA and 0.1 percent Tween-20. Incubation was overnight at about 25 4°C with rocking. After incubation, the Western blot was washed three times in TBST for about 5 minutes per wash with rocking at room temperature.

30

35

The secondary antibody used to detect beta-secretase antibody was alkaline phosphatase conjugated 40 Goat Anti-rabbit IgG (Promega, Madison, WI; item number S373B). This antibody was diluted at about 1:7500 in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.2, 100 mM sodium chloride, 5 mM MgCl₂) and added to the 45 Western blot. After about 30 minutes of incubation at 50 room temperature, the Western blot was washed as described above and developed about 5-10 minutes with BCIP/NBT (Kirkegaard and Perry Labs, Gaithersburg, MD;

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- 102 -

item number 50-81-08). To stop development, the
10 Western blot was rinsed in stop solution (20 mM Tris-HCl, pH 8.0, 5 mM EDTA) and dried.

The results are shown in Figure 8. "P"
15 represents immunoprecipitations with preimmune serum, and "A" represents immunoprecipitations with anti-beta secretase antibody. "Cells" refers to human cultured cells transfected with the human beta-secretase cDNA; 20 "AD" refers to brain tissue homogenate from Alzheimer's disease patients; and "Control" refers to brain tissue homogenate from normal people. As can be seen, a major band of approximately 70 kilo daltons is apparent in lane 2. A band of approximately the same molecular weight is present in lanes 4 and 6 as well. This 25 result indicates that the transfected cells are expressing the beta secretase protein which is observed in human brain. The molecular weight of beta-secretase calculated from the amino acid sequence is about 55.8 30 kilo daltons, suggesting that the protein expressed in 35 cells and brain could be glycosylated.
40

Example VIII: Preparation of a Human Beta-Secretase

45 Mutant

25

A hemagglutinin (HA) epitope tag was added in frame to the C-terminus of the full length beta-

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- 103 -

10 secretase cDNA for the purpose of detection with the
monoclonal anti-HA antibody HA.11 (obtained from BabCo,
Berkeley CA). The HA epitope tag consists of 11 amino
acids and was used as a marker tag, enabling the
15 surveillance of the beta-secretase-HA fusion
polypeptide via anti-HA antibody detection.

20 The C-terminal HA tagged form of beta-
secretase was generated by PCR in two steps. For the
first step, the forward primer had 5 prime to 3 prime
10 sequence corresponding to nucleotides 1047-1070 of the
beta-secretase cDNA and had the sequence:
25

TGACTCTCTGGTAAAGCAGACCCA (SEQ ID NO:8)

30 35 The reverse primer for this first round of
PCR corresponded to nucleotides 1938-1957 of beta-
secretase together with the first 18 base pairs of the
HA tag and had the sequence:

40 20 AGGCACGTCGTAAGGGTACTTCAGCAGGGAGATGTCA (SEQ ID NO:9)

45 The PCR conditions for this reaction were:
97C for 60 seconds; 97C for 30 seconds, 60C for 30
seconds and 72C for 1 minute for twenty five cycles.
50 25 This round of PCR generated a 928 base pair product.
This PCR product was then purified using the Wizard™
PCR Preps DNA Purification System (Promega, Madison,

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- 104 -

10 WI). This PCR product was then used as template for
the second PCR reaction. This reaction used the
identical forward primer and a reverse primer
corresponding to all 11 amino acids of the HA tag
15 followed immediately by a termination codon and a *BglII*
restriction site. This primer had the sequence:

20 TGAAGATTTCATCCGCTGGCATAATCAGGCACGTCTAAGGGTA (SEQ ID
NO:10)

10
25 PCR conditions for this reaction were
identical to those used for the first reaction. The
955 base pair PCR product that resulted from this
30 second round of PCR was purified as described above and
15 digested with *BsrGI* and *BglII* overnight at 37C. The
digested fragment was gel purified from a 0.8 percent
agarose gel using the QiaQuick gel purification system
35 (Qiagen, Chatsworth, CA) and subsequently subcloned
into *BsrGI/BglII* digested pCMVi-beta-secretase.

20 A mutant DNA construct encoding D93A human
40 beta secretase was prepared via PCR. Each fragment
contained the D93A mutation and 24 base pairs of
overlapping sequence. The first or forward fragment
45 contained the D93A mutation in the sense orientation at
25 the extreme 3 prime end of the fragment, while the
second or reverse fragment contained the D93A mutation
50 at the extreme 5 prime end of the fragment in the

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- 105 -

10 antisense orientation. Next, these PCR products were purified and combined together (at equimolar ratio) in a second amplification reaction using just the two outer primers for amplification, thus giving rise to a
15 5 full length double stranded 953 bp *BamHI-BsrGI 008* fragment of beta secretase containing the D93A mutation. This PCR product was then subsequently used
20 to replace the *BamHI-BsrGI* fragment of the native beta-secretase cDNA.

10 The forward first step PCR product was
25 generated using a 5 prime forward primer which is identical to bases 211-233 of the beta-secretase cDNA sequence. This primer has the sequence:

30 15 GTGCCGATGTAGCGGGCTCCGGA (SEQ ID NO:11)

35 A 3 prime reverse primer was generated which corresponds to bases 720-743 of human beta-secretase
40 20 cDNA except that the T at position 731 was changed to G thus changing the aspartic acid residue at position 93 of the peptide sequence to an alanine residue (on the antisense strand). This primer has the sequence:

45 CTGCTGCCTGTAGCCACCAAGGATG (SEQ ID NO:12)

50 25 The reverse first step PCR product was generated using a 5 prime forward primer in which the A

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- 106 -

10 at base 731 was changed to a C, thereby creating a construct that encoded an alanine at position 93 of the human beta-secretase polypeptide sequence (on the sense strand). This primer has the sequence:

15

5 CATCCTGGTGGCTACAGGCAGCAG (SEQ ID NO:13)

20

The 3 prime reverse primer used in this PCR reaction is identical to bases 1230-1254 of the beta secretase human cDNA. This primer has the sequence:

25

CACCCGCACAATGATCACCTCATAA (SEQ ID NO:14)

30

PCR reactions for the forward first step reaction were conducted in an MJ Research PTC 200 thermal cycler (MJ Research, Watertown, MA) using the following conditions: 97C for 90 seconds; 98C for 30 seconds, 60C for 30 seconds and 72C for 1 minute for twenty five cycles. The two PCR products were then purified using the Wizard™ PCR Preps DNA Purification System (Promega, Madison, WI).

45

For the second step PCR reaction, the two PCR fragments generated above were added in an approximately equimolar ratio, and were subsequently amplified using the following primers:

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- 107 -

GTGCCGATGTAGCGGGCTCCGGA (SEQ ID NO:15)

10

CACCCGCACAATGATCACCTCATAA (SEQ ID NO:16)

15

The PCR conditions for this reaction were identical to those used in the first step reactions. The PCR products were purified using the Wizard™ PCR Preps DNA Purification System (Promega, Madison, WI), restriction digested with *BamHI* and *BsrG,I* and gel purified on a 0.8 percent agarose gel using the QiaQuick gel purification system (Qiagen, Chatsworth, CA). This fragment was then subcloned into the *BamHI/BsrGI* digested pCMVi-beta-secretase/ HA tag construct.

30

In a separate procedure, the human APP-695 cDNA was subcloned into the CMV based expression vector pCMVi (Cell & Molecular Technologies Inc., LaVallette, New Jersey) as a *HindIII-NotI* fragment to generate the vector pCMVi-APPwt. A derivative of 293 cells containing the SV40 large T antigen (Cell & Molecular Technologies, LaVallette, NJ) maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 percent fetal calf serum was co-transfected with pCMVi-APPwt and pRSV-Puro by the calcium phosphate method as described (Gorman et al., *supra*). Transfected cells were cultured in medium containing puromycin at a concentration of about 5

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- 108 -

10 micrograms/ml . Puromycin resistant colonies were selected about 48 hours after transfection. Fourteen days later, individual resistant colonies were isolated, expanded, and analyzed by Western dot blot
15 5 for APP expression using the monoclonal antibody 22C11 (Boehringer Manheim Corp.) which recognizes secreted APP.

20 One positive colony was expanded to generate a cell line referred to as "293T/APPwt".

10 To assess the activity of the D93A mutant,
25 293T/APPwt cells were transfected with beta-secretase cDNA (either wild type or D93A mutant) or with control plasmid (no beta-secretase insert) as follows.

30 The 293T/APPwt cells were plated out at a
35 15 density of about 2.8×10^3 cells per well in a 6-well tissue culture plate (Falcon) and grown for 2 days at 37°C in an atmosphere of about 5 percent CO₂. The cells
40 20 were transfected the morning of day 3. Prior to transfection, all plasmids to be used were resuspended
45 25 in water. Two polystyrene tubes were set up for each well. One tube received about 3 µg total DNA (control plasmid was diluted 1:10 as compared with the plasmids containing beta-secretase inserts) and 1 ml of Opti-MEM I reduced serum media (Gibco-BRL, Grand Island, New York). The second tube received about 14 µl DMRIE-C (Gibco-BRL) and 1 ml Opti-MEM I. The tubes were then
50 25 mixed together and incubated at room temperature for

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- 109 -

about 30 minutes. The media was then removed from the
10 cells and the DNA/ DMRIE-C/ Opti-MEM I mixture (2 ml
total volume) was added to each well. The cells were
15 incubated at about 37°C in about 5 percent CO₂ for about
5 hours. Following the incubation, about 2 ml of Opti-
MEM I containing 20 percent fetal bovine serum (Gibco-
BRL) were added to each well of cells, after which the
20 cells were incubated at about 37°C, in an atmosphere of
about 5 percent CO₂, overnight. The following morning
10 (day 4) the media was removed and 2 ml of fresh DMEM
25 containing 10 percent fetal bovine serum (Gibco-BRL)
were added, and the cells were then incubated at about
37°C in an atmosphere of about 5 percent CO₂, overnight.
The following day (day 5) the conditioned media was
30 15 collected, and sodium dodecyl sulfate (Sigma, St.
Louis, MO) was added to a final concentration of about
0.1 percent. The samples were stored at about -80°C.

35 Figure 9A and 9B show the results of the
transfection. Conditioned media of cells transfected
20 with the wild type beta-secretase expression plasmid
contain significantly higher levels of A-beta 42 (Fig.
9A) and A-beta 40 (Fig. 9B) than conditioned media of
40 cells transfected with either the control plasmid or
conditioned media of cells transfected with the beta-
45 secretase D93A-HA mutant. This suggests that D93 is
25 part of the active site of human beta-secretase.

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- 110 -

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Example IX: Purification of Soluble Beta Secretase-Fc
Fusion Polypeptide

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A human IgG1 Fc gene (the sequence of which
is set forth in Genbank as accession number X70421) was
amplified by polymerase chain reaction and subcloned
into the vector pBluescript SK- (Stratagene, La Jolla,
CA). PCR was conducted using a MJ Research PTC-200
thermal cycler for 25 cycles with the following
parameters: 94C for 1 minute; 55C for 1 minute; and 72C
for 1 minute. The polymerase used was Pfu polymerase
(Stratagene, La Jolla, CA). The following primers were
used as forward and reverse primers, respectively for
this PCR reaction:

20

15

CGGGATCCGGTCACCGACAAACTCACACA (SEQ ID NO:20)

25

GCTCTAGAAGCTTCTGCAGGTCGACTCATTACCCGGAGA (SEQ ID NO:21)

30

The forward primer incorporated 5' *BamHI* and
BstEII sites at the 5' end of the PCR product, and the
reverse primer incorporated *SalI*, *PstI*, *HindIII*, and
XbaI sites at the 3' end of the 723 base pair PCR
fragment that was generated using these primers. The
PCR fragment was purified using Qiaquick™ purification
kit (Qiagen, Chatsworth, CA), and digested with *BamHI*
and *XbaI*. After a second round of gel purification,

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- 111 -

the PCR fragment was ligated into the vector
10 pBluescript SK- (Stratagene, La Jolla, CA) that was
previously digested with *BamHI* and *XbaI*.

An 1195 base pair fragment of the human beta-
15 secretase cDNA (nucleotides 276 to 1471) was excised
from the full length cDNA by digestion with *EcoRI* and
BsrGI and gel purified. This fragment contained the 5'
20 untranslated region and the coding region up to amino
acid 245 of the human beta secretase cDNA. A second
10 fragment of human beta-secretase cDNA encoding amino
acids 246 to 460 (ending exactly at the start of the
25 putative transmembrane domain) was amplified by PCR
using procedures described above for the IgG1 fusion
gene. Amplification forward and reverse primers for
30 15 this reaction were as follows:

CGACCACTCGCTGTACACAGGCAG (SEQ ID NO:22)

35

GTCGGTGACCGCATAGGCTATGGTCATGAGGGT (SEQ ID NO:23)

20

40 The forward primer incorporated the
endogenous 5' *BsrGI* site, and the reverse primer
incorporated a 3' *EstEII* site for in-frame ligation of
45 the PCR product into the IgG1 fusion gene cassette.
25 The resulting 672 base pair PCR fragment was purified
using the Qiaquick™ purification kit (Qiagen,

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- 112 -

10 Chatsworth, CA), digested with *BsrGI* and *BstEII*, and
gel-purified.

15 Separately, the human IgG1 fusion cassette
was digested with *EcoRI* and *BstEII* and gel-purified. A
5 3-way ligation was then conducted by combining the 1195
base pair *EcoRI/BsrGI* human beta-secretase fragment and
the 672 base pair *BsrGI/BstEII* beta secretase PCR
fragment with the *EcoRI/BstEII* human IgG1 fusion
20 cassette. The completed beta secretase fusion gene was
10 called beta-secretase-Fc and contained 3 additional
amino acids (alanine-valine-threonine) at the junction
25 between the beta-secretase and IgG1 coding regions.

30 The beta-secretase-Fc fusion gene was excised
from the plasmid pBluescript SK- by digestion with *XhoI*
35 and *NotI*, and gel-purified. The mammalian expression
15 vector pCMVi (Cell & Molecular Technologies Inc.
Lavallette, NJ) was also digested with the same enzymes
and gel-purified, after which the beta-secretase-Fc
50 fusion gene fragment was ligated into the *XhoI/NotI*
20 digested pCMVi vector.

40 Human embryonic kidney 293T cells (Cell &
Molecular Technologies Inc., Lavallette, NJ) stably
transfected with the beta-secretase-Fc fusion construct
45 by the standard calcium phosphate method (Gorman et al,
25 DNA Prot. Eng. Tech. 2: 3-10 [1990]) were conditioned
for either four or five days in Opti-Mem reduced serum
50 medium (GibcoBRL, Grand Island, NY; item number 31985).

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- 113 -

10 After filtration with a 0.45 µm filter, conditioned
media was concentrated and buffer exchanged to column
equilibration buffer (PBS containing 2mM Chaps). The
concentrated conditioned medium was then loaded onto a
15 5 milliliter recombinant protein A column (Amersham
Pharmacia Biotech, Inc, Piscataway, NJ; item number 17-
5079-02) which had been pre-equilibrated with 10 column
20 volumes of PBS containing 2mM Chaps™ at about 4C.

25 After loading the sample on the column, the column was
washed with 10 column volumes of column equilibration
buffer, and the beta-secretase-Fc fusion protein was
eluted from the column with a step gradient to 100 mM
30 Sodium Citrate, pH 2.8 containing 2mM Chaps™. One
milliliter fractions were collected. Before
35 collection, fraction collection tubes were loaded with
300 ml of 1M Tris base, pH 9.2, to neutralize fractions
upon elution. Final pH of each 1.3 ml fraction was
approximately pH 7.5. Fractions containing beta-
secretase-Fc fusion polypeptide were pooled,
40 concentrated, and buffer exchanged to TBS containing 2
mM Chaps for storage.

45 Figure 10 shows a Coomassie stained SDS gel
of the purified beta-secretase-Fc fusion protein (lane
2) alongside molecular weight markers (lane 1). Only
50 25 the beta-secretase-Fc fusion polypeptide and some
cleaved Fc polypeptide were detectable. The predicted

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- 114 -

10 molecular weight of the fusion protein is 76.8 kilo
daltons. The higher observed molecular weight is
presumably due to glycosylation.

15 5 Example X: Beta-Secretase Activity Assay

20 Recombinant native as well as mutant beta
secretase polypeptides were assayed for activity by
measuring the ability of the molecule to cleave various
10 A-beta peptides.

25 All peptides were synthesized by the Fmoc
(fluorenylmethoxycarbonyl) / t-butyl based solid phase
peptide chemistry method using standard procedures. An
ABI 431A peptide synthesizer (Perkin Elmer Corp.,
30 Foster City, CA) was used with a single coupling
program to carry out the chain assembly. Commercially
available preloaded Fmoc-AAA-HMP derivatized
35 polystyrene resin (Midwest Biotech, Fishers, IN or
Calbiochem, San Diego, CA) was used to prepare the
20 C-terminal amino acid. Subsequent amino acids were
coupled in 20 fold excess as HOBT (hydroxybenztriazole)
40 esters using carbodiimide activation. The side-chain
protecting groups for each amino acid were as follows:
Arg(Pbf; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-
45 sulfonyl), Asn(Trt; trityl), Asp(OtBu; O-tert-butyl),
Cys(Trt; trityl), Cys(Acm; acetamidomethyl), Gln(Trt;
50 trityl), Glu(OtBu; O-tert-butyl), His(Trt; trityl),

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- 115 -

10 Lys(Boc; tert-butoxycarbonyl), Ser(tBu; tert-butyl),
15 Thr(tBu), and Tyr(tBu; tert-butyl). Upon removal of
the final N-terminal Fmoc with 20 percent piperidine in
N-methylpyrrolidone, side-chain protecting groups were
20 5 then removed and the peptide(s) were cleaved from the
resin by treatment with TFA (trifluoroacetic acid):
25 triisopropylsilane: water (92.5, 2.5, 5 v/v) for about
4 hours. The resulting suspension was filtered, and
the filtrate volume reduced by roto-evaporation. The
10 crude peptides were precipitated and washed with ether,
followed by drying in-vacuo.

25 The linear (fully reduced) peptide
30 intermediates were purified by HPLC prior to either an
equilibrium oxidative refold or a two step oxidative
35 15 cyclization process. The connectivity of those
peptides containing one or two disulfides is
unambiguous as a result of using orthogonal cysteine
protection and oxidative-cyclization methods as
40 follows. The first disulfide bond was formed using air
45 oxidation (Cys[Trt] protection) and the second
disulfide bond was formed by iodine treatment on the
bis-Cys(Acm) containing -cyclic peptide intermediate.
50 For the two peptides containing three disulfide
bridges, oxidation was carried out using 10 percent
55 25 DMSO (dimethylsulfoxide) oxidation under acidic
conditions.

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- 116 -

10 Each crude peptide was dissolved in 8M
guanidine containing 100 mM DTT (dithiothreitol) and
purified to at least 95 percent homogeneity by
preparative reverse-phase HPLC using a Vydac C₁₈ (2.5
15 5 cm x 25 cm) column (Vydac Corp., Hesperia, CA) with a
linear gradient of 0.1 percent TFA (v/v) in water and
0.05 percent TFA (v/v) in acetonitrile.

20 The composition of each peptide was assessed
using electro-spray ionization (ESI) mass spectrometry
10 and amino acid analysis. Mass spectra for each
synthetic peptide was obtained on a Sciex API (Perkin
25 Elmer Corp., Foster City, CA) single quadrupole mass
spectrometer, and reported as *m/z* (M+1). All mass
spectral samples were obtained as fractions off of the
30 preparative HPLC purification.

35 Amino acid analyses of each peptide were
performed on an ABI 420A hydrolyzer/derivatizer (ABI,
Foster City, CA) using a 130A separation system (ABI,
Foster City, CA). The peptides were hydrolyzed using
40 6N HCl at 200° for 30 minutes and then derivatized
using the defined instrument protocol in the ABI
AAA420A Operator's Manual as PTC (phenylisothiocyanate)
45 derivatives. The amino acid mixture was then separated
by HPLC on a Brownlee PTC C₁₈ column (ABI, Foster City,
50 CA). 5 micron pore size, 2.1 x 220 mm, with a linear
gradient of water and acetonitrile. Both solvents were

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- 117 -

10 buffered with sodium acetate to a pH of about 5.4. The
amino acid composition of each peptide was then
determined by comparison of the unknown peak ratios
with an equimolar amino acid standard. Each peptide
15 5 generated experimental data that conformed with
expected theoretical values.

20 The sequence of the synthesized peptides
(from amino to carboxy terminus) is set forth in Table
III below:

10

25

Table III

30

| Peptide | Peptide Sequence | |
|---------|------------------|--------------|
| Sw | EVNLDAEF | SEQ ID NO:17 |
| WT | EVKMDAEG | SEQ ID NO:18 |
| MV | EVKVDAEF | SEQ ID NO:19 |

35

40 To facilitate analysis of the assays, all of
15 the peptides were labeled with dinitrophenol (DNP) at
the amino terminus.

45

DNP was incorporated during synthesis of the
peptide substrates as dnp-glutamic acid.

50

Each assay was conducted as follows. The
20 purified beta secretase-FC (amounts indicated in Fig.
11) was combined separately with each peptide substrate

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- 118 -

(20 μ M) to a final volume of about 50 μ l in a buffer
10 containing 50 mM acetic acid, 50 mM Mes, 100 mM Tris,
pH 5.0 and 0.05 percent (w/v) Thesit. The samples were
15 incubated at ambient room temperature for about 6
hours. At the end of the incubation period, reaction
mixtures were quenched by addition of about 200 μ l of 5
percent (v/v) trifluoroacetic acid (TFA). Quenched
reactions were analyzed by HPLC on a 5 μ m, C18
20 reversed-phase column (Sephadex Peptide, 4.6 x 100 mm;
10 Amersham Pharmacia Biotech, Piscataway, NJ) using first
25 a linear gradient of 18-31.5 percent acetonitrile in
0.1 percent TFA over 40 minutes, then 31.5-81 percent
acetonitrile for 1 minute, then 81 percent acetonitrile
30 for 4 minutes, and then returning to 18 percent
15 acetonitrile for several minutes to reequilibrate the
column. The linear 18-31.5 percent acetonitrile
gradient was subsequently modified to a concave
35 gradient (curve 7 as defined by the Dionex GP40 pump
system, Dionex Corporation, Sunnyvale, CA) from 18-31.5
20 percent acetonitrile in 15 minutes. Proteolysis of
the Sw and WT octapeptides was evaluated using a linear
40 gradient of 27-37.8 percent acetonitrile over 24
minutes on the same column.
45 Both product and substrate were monitored by
25 absorbance at 360 nm. Products were typically
identified by retention time comparison with
50 appropriate substrate peptides run under identical

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- 119 -

10 conditions. The rate of product formation or the amount of product formed was determined by comparison of the area under the product peak to a standard curve of authentic peptide. The reference product peptides
15 5 were each labeled with dinitrophenol at the amino terminus, and had the sequences:

20 EVNL (SEQ ID NO:24)
EVKV (SEQ ID NO:25)
10 EVKM (SEQ ID NO:26)

25 The concentration of standard was determined from the extinction coefficient of DNP ($e_{365\text{nm}} = 16 \text{ mM}^{-1}\text{cm}^{-1}$). The HPLC assay was linear and reproducible from
30 15 approximately 25 to about 300 pmol of product.

35 The results are shown in Figure 11 for each substrate. As can be seen, the Swedish substrate (Sw) is clearly preferred by the enzyme over the wild type (Wt)
40 20 substrate which is preferred over the methionine to valine mutant (MV) substrate. These results indicate that purified beta-secretase-Fc has the same substrate specificity that has previously been described for beta-secretase from intact cells (Citron et al., *Neuron* 14:661-670 91995]).

45 25 While the present invention has been described in terms of the preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended
50 30 that the appended claims cover all such equivalent

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- 120 -

**variations which come within the scope of the invention
as claimed.**

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Claims

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- 121 -

WHAT IS CLAIMED IS:

- 10 1. An isolated nucleic acid molecule selected
from the group consisting of:
5 a) the nucleic acid molecule as set forth in
any of SEQ ID NOS: 1, 2, and 3;
15 b) a nucleic acid molecule encoding the
polypeptide of any of SEQ ID NOS: 4, 5, and 6;
10 c) biologically active fragments of SEQ ID
NO:4;
20 d) an allelic variant or splice variant of
any of (a) or (b);
25 e) a nucleic acid molecule of the DNA vector
insert in ATCC Deposit No. 207158;
15 f) a nucleic acid molecule of the DNA vector
insert in ATCC Deposit No. 207159;
30 g) a nucleic acid molecule encoding a
polypeptide having one to fifty conservative amino acid
substitutions as compared with the polypeptide of SEQ
20 ID NO:4, wherein the polypeptide encoded by said
nucleic acid molecule is biologically active; and
35 h) a nucleic acid molecule that is the
complement of any of (a)-(g) above.
- 35 2. A nucleic acid molecule that is SEQ ID
NO:1.
- 40 3. A nucleic acid molecule encoding the
polypeptide of SEQ ID NO:4.
- 30 4. A nucleic acid molecule encoding the
biologically active polypeptide fragment of claim 13.
- 45 5. An expression vector comprising the
35 nucleic acid molecule of Claim 1.

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- 122 -

6. A host cell comprising the expression
vector of Claim 5.

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7. The host cell of Claim 6 which is a
5 eukaryotic cell.

15

8. The host cell of Claim 6 which is a
prokaryotic cell.

20

9. A process for producing a beta secretase
polypeptide comprising culturing the host cell of Claim
6 in suitable culture medium and isolating the
polypeptide from the culture.

25

10. A polypeptide produced by the process of
Claim 9.

30

11. An isolated polypeptide selected from the
group consisting of:

- 20 a) the polypeptide of any of SEQ ID NOS: 4,
5, and 6;
b) a biologically active fragment of any of
SEQ ID NOS. 4, 5, 6;
c) a biologically active polypeptide having
25 one to fifty conservative amino acid changes as
compared with the polypeptide of SEQ ID NO:4;
d) the polypeptide encoded by the DNA vector
40 insert of ATCC Deposit Nos. 207158 and 207159; and
e) a polypeptide that is an allelic variant
30 or splice variant of (a).

45

12. An isolated polypeptide encoded by the
nucleic acid molecule of Claim 1.

50

35 13. An isolated polypeptide having the amino
acid sequence of SEQ ID NO:4.

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- 123 -

10 14. An isolated beta secretase polypeptide
fragment of SEQ ID NO:4 selected from the group
consisting of: amino acids 45-501; amino acids 46-501;
5 amino acids 46-460; amino acids 45-460; amino acids 1-
460; amino acids 93-292; amino acids 93-293; amino
15 acids 91-295; amino acids 90-295; amino acids 90-300;
 amino acids 62-420; amino acids 1-420; amino acids 62-
460; amino acids 90-460; amino acids 62-501; amino
10 acids 62-460; amino acids 90-293; amino acids 90-300;
 amino acids 1-420; amino acids 46-420; amino acids 62-
20 420; amino acids 73-420; amino acids 83-420; amino
 acids 90-420; amino acids 62-417; amino acids 73-417;
 amino acids 83-417; amino acids 90-417; amino acids 62-
25 410; amino acids 73-410; amino acids 83-410; amino
 acids 90-410; amino acids 62-402; amino acids 73-402;
 amino acids 83-402; and amino acids 90-402.

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20 15. An antibody or fragment thereof
specifically binding the polypeptide of Claim 10.

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16. The antibody of Claim 15 that is a
monoclonal antibody.

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25 17. A composition comprising the polypeptide
of Claim 10 and a pharmaceutically acceptable
formulation agent.

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30 18. A polypeptide comprising a derivative of
the polypeptide of Claim 11.

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35 19. A fusion polypeptide comprising the
polypeptide of Claim 11 fused to a heterologous amino
acid sequence.

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- 124 -

10 20. The fusion polypeptide of Claim 18
wherein the heterologous amino acid sequence is an IgG

constant domain or fragment thereof.

15 5 21. A method of modulating levels of beta
secretase polypeptide in a mammal comprising

administering to the animal the nucleic acid molecule
of Claim 1.

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FIG. 1A

ATGGCCCAAG CCCTGCCCTG GCTCCTGCTG TGGATGGCG CGGGAGTGCT
GCCTGCCAC GGCACCCAGC ACGGCATCCG GCTGCCCTG CGCAGCGGCC
TGGGGGGCGC CCCCCCTGGGG CTGCGGCTGC CCCGGGAGAC CGACGAAGAG
CCCGAGGAGC CCGGCCGGAG GGGCAGCTTT GTGGAGATGG TGGACAACCT
GAGGGGCAAG TCAGGGCAGG GCTACTACGT GGAGATGACC GTGGGCAGCC
CCCCCGAGAC GCTAACATC CTGGTGGATA CAGGCAGCAG TAACTTTGCA
GTGGGTGCTG CCCCCCACCC CTCCTGCAT CGCTACTACC AGAGGCAGCT
GTCCAGCACA TACCGGGACC TCCGGAAGGG TGTGTATGTG CCCTACACCC
AGGGCAAGTG GGAAGGGAG CTGGGCACCG ACCTGGTAAG CATCCCCAT
GGCCCCAACG TCACTGTGCG TGCCAACATT GCTGCCATCA CTGAATCAGA
CAAGTTCTTC ATCAACGGCT CCAACTGGGA AGGCATCCTG GGGCTGGCCT
ATGCTGAGAT TGCCAGGCCT GACGACTCCC TGGAGCCTTT CTTTGACTCT
CTGGTAAAGC AGACCCACGT TCCCAACCTC TTCTCCCTGC AGCTTTGTGG
TGCTGGCTTC CCCCTCAACC AGTCTGAAGT GCTGGCCTCT GTGGAGGGGA
GCATGATCAT TGGAGGTATC GACCACTCGC TGTACACAGG CAGTCTCTGG
TATACACCCA TCCGGGGGA GTGGTATTAT GAGGTGATCA TTGTGCGGGT
GGAGATCAAT GGACAGGATC TGAAAATGGA CTGCAAGGAG TACAACATATG
ACAAGAGCAT TGTGGACAGT GGCACCCACCA ACCTTCGTTT GCCCAAGAAA
GTGTTGAAG CTGCAGTCAA ATCCATCAAG GCAGCCTCCT CCACGGAGAA
GTTCCCTGAT GGTTCTGGC TAGGAGAGCA GCTGGTGTGC TGGCAAGCAG
GCACCCACCC TTGGAACATT TTCCCAAGTCA TCTCACTCTA CCTAATGGGT
GAGGTTACCA ACCAGTCCTT CCGCATTACCC ATCCTCCGC AGCAATACCT
GCAGGCCAGTG GAAGATGTGG CCACGTCCCA AGACGACTGT TACAAGTTG

FIG. 1B

CCATCTCACA GTCATCCACG GGCACTGTTA TGGGAGCTGT TATCATGGAG
GGCTTCTACG TTGTCTTGAA TCAGGGCCCGA AAACGAATTG GCTTTGCTGT
CAGCGCTTGC CATGTGCACG ATGAGTTCAAG GACGGCAGCG GTGGAAGGCC
CTTTTGTAC CTTGGACATG GAAGACTGTG GCTACAAACAT TCCACAGACA
GATGAGTCAA CCCTCATGAC CATAGCCTAT GTCATGGCTG CCATCTGCGC
CCTCTTCATG CTGCCACTCT GCCTCATGGT GTGTCAGTGG CGCTGCCTCC
GCTGCCTGCG CCAGCAGCAT GATGACTTTG CTGATGACAT CTCCCTGCTG
AAG

FIG. 2A

ATGGCCCCAG CGCTGCACTG GCTCCTGCTA TGGGTGGGCT CGGGAATGCT
GCCTGCCAG GGAACCCATC TCGGCATCCG GCTGCCCTT CGCAGCGGCC
TGGCAGGGCC ACCCCTGGC CTGAGGCTGC CCCGGGAGAC CGACGAGGAA
TCGGAGGAGC CTGGCCGGAG AGGCAGCTTT GTGGAGATGG TGGACAACCT
GAGGGGAAAG TCCGGCCAGG GCTACTATGT GGAGATGACC GTAGGCAGCC
CCCCACAGAC GCTAACATC CTGGTGGACA CGGGCAGTAG TAACTTGCA
GTGGGGCTG CCCCACACCC TTTCCTGCAT CGCTACTACC AGAGGCAGCT
GTCCAGCACA TATCGAGACC TCCGAAAGGG TGTGTATGTG CCCTACACCC
AGGGCAAGTG GGAGGGGAA CTGGGCACCG ACCTGGTGAG CATCCCTCAT
GGCCCCAACG TCACTGTGCG TGCCAACATT GCTGCCATCA CTGAATCGGA
CAAGTTCTTC ATCAATGGTT CCAACTGGGA GGGCATCCTA GGGCTGGCCT
ATGCTGAGAT TGCCAGGCC GACGACTCTT TGGAGCCCTT CTTTGACTCC
CTGGTGAAGC AGACCCACAT TCCCAACATC TTTCCCTGC AGCTCTGTGG
CGCTGGCTTC CCCCTCAACC AGACCGAGGC ACTGGCCTCG GTGGGAGGGA
GCATGATCAT TGGTGGTATC GACCACTCGC TATAACACGGG CAGTCTCTGG
TACACACCCA TCCGGCGGGGA GTGGTATTAT GAAGTGATCA TTGTACGTGT
GGAAATCAAT GGTCAAGATC TCAAGATGGA CTGCAAGGAG TACAACACTACG
ACAAGAGCAT TGTGGACAGT GGGACCACCA ACCTTCGCTT GCCCAAGAAA
GTATTGAAAG CTGCCGTCAA GTCCATCAAG GCAGCCTCCT CGACGGAGAA
GTTCCCGGAT GGCTTTGGC TAGGGGAGCA GCTGGTGTGC TGGCAAGCAG
GCACGACCCC TTGGAACATT TTCCCAGTCA TTTCACTTTA CCTCATGGGT
GAAGTCACCA ATCAGTCCTT CCGCATCACC ATCCTTCCTC AGCAATACCT
ACGGCCGGTG GAGGACGTGG CCACGTCCCA AGACGACTGT TACAAGTTCG
CTGTCTCACCA GTCATCCACG GGCACGTGTTA TGGGAGCCGT CATCATGGAA

FIG. 2B

GGTTTCTATG TCGTCTTCGA TCGAGCCCGA AAGCGAATTG GCTTTGCTGT
CAGCGCTTGC CATGTGCACG ATGAGTTCA GACGGCGGCA GTGGAAGGTC
CGTTTGTAC GGCAGACATG GAAGACTGTG GCTACAACAT TCCCCAGACA
GATGAGTCAA CACTTATGAC CATAGCCTAT GTCATGGCGG CCATCTGCGC
CCTCTTCATG TTGCCACTCT GCCTCATGGT ATGTCAGTGG CGCTGCCTGC
GTTGCCTGCG CCACCAGCAC GATGACTTTG CTGATGACAT CTCCCTGCTC
AAG

FIG. 3A

ATGGCCCCGG CGCTGCGCTG GCTCCTGCTA TGGGTGGGCT CGGGAATGCT
GCCTGCCAG GGAACCCATC TCGGTATCCG ACTGCCCTT CGCAGCGGCC
TGGCAGGGCC ACCCCTGGC CTGAGGCTGC CCCGGGAGAC GGACGAGGAA
CCTGAGGAGC CTGGCCGGAG AGGCAGCTTT GTGGAGATGG TGGACAACCT
GAGGGGAAAG TCCGGCCAGG GCTACTATGT GGAGATGACC GTGGGCAGCC
CCCCACAGAC GCTAACATC CTGGTGGACA CGGGCAGTAG TAATTTGCA
GTGGGGGCTG CCCCACACCC TTTCCTGCAT CGATACTACC AAAGGCAGCT
GTCCAGTACA TACCGAGACC TCCGAAAGTC TGTGTATGTG CCCTACACCC
AGGGCAAGTG GGAGGGGAA CTGGGCACTG ACCTGGTGAG CATCCCTCAT
GGCCCCAACG TCACTGTGCG TGCCAACATT GCTGCCATCA CTGAATCGGA
CAAGTTCTTC ATCAATGGTT CCAACTGGGA GGGCATCCTA GGGCTGGCCT
ATGCTGAGAT TGCCAGGCCT GACGACTCCT TGGAGCCCTT TTTTGACTCC
CTGGTGAAGC AGACCCACAT TCCGAACATC TTTCCCTGC AGCTCTGTGG
CGCTGGCTTC CCCCTCAACC AGACTGAGGC ACTGGCCTCG GTGGGAGGGA
GCATGATCAT TGGTGGTATC GACCATTCCC TATAACTGG CAGTCTCTGG
TACACACCCA TCCGGCGGGA GTGGTATTAT GAAGTGATCA TTGTACGTGT
AGAAAATCAAT GGTCAAGATC TGAAAATGGA CTGCAAGGAG TACAACATATG
ACAAGAGCAT CGTGGACAGT GGCACCACCA ACCTTCGTTT GCCCAAGAAA
GTATTTGAAG CTGCAGTCAA GTCCATCAAG GCAGCCTCCT CGACGGAGAA
GTTCCCGGAT GGCTTTGGC TAGGGGAGCA GCTGGTGTGC TGGCAAGCAG
GCACGACCCC TTGGAACATT TTCCCAGTCA TTTCACTTTA CCTCATGGGT
GAAGTCACCA ATCAGTCCTT CCGCATCACCA ATCCTTCCTC AGCAATACT
ACGGCCAGTG GAAGATGTGG CCACGTCCCCA AGACGACTGT TACAAGTTCG

FIG. 3B

CCGTCTCACA GTCATCCACA GGCACCGTTA TGGGAGCGGT CATCATGGAA
GGCTTCTATG TGGTCTTGAG TCGAGCCCGA AAGCGAATTG GCTTTGCTGT
CAGCGCTTGC CATGTGCACG ATGAGTTCAAG GACGGCGGCA GTGGAAGGTC
CGTTTGTACAC GGCAGACATG GAAGACTGTG GCTACAAACAT TCCACAGACAGA
GATGAGTCAA CACTTATGAC CATAGCCTAT GTCATGGCTG CCATCTGCGC
CCTCTTCATG TTGCCACTCT GCCTCATGGT ATGTCAGTGG CGCTGCCTAC
GCTGCCTGCG CCATCAGCAT GATGACTTTG CTGATGACAT CTCCCTGCTG
AAA

FIG. 4

MAQALPWLLL WMGAGVLPAA GTQHNGIRLPL RSGLGGAPLG LRLPRETDEE
PEEPGRGSF VEMVDNLRGK SGQGYYVEMT VGSPPQTLNI LVDTGSSNFA
VGAAPHFPLH RYYQRQLSST YRDLRKGVYV PYTQGKWEGE LGTDLVSIPH
GPNVTVRANI AAITESDKFF INGSNWEGIL GLAYAEIARP DDSLEPFFDS
LVKQTHVPNL FSLQLCGAGF PLNQSEVLAS VGGSMIIGGI DHSLYTGSLW
YTPIRREWYY EVIIVRVEIN GQDLKMDCKE YNYDKSIVDS GTTNLRLPKK
VFEAAVKSIA AASSTEKFDP GFWLGEQLVC WQAGTPWNI FPVISLYLMG
EVTNQSFRIT ILPQQYLRPV EDVATSQDDC YKFAISQSST GTVMGAVIME
GFYVVFDRAR KRIGFAVSAC HVHDEFRTAA VEGPFVTLDM EDCGYNIPQT
DESTLMTIAY VMAAICALFM LPLCLMVCQW RCLRCLRQQH DDFADDISLL
K

FIG. 5

MAPALHWLLL WVSGGMLPAQ GTHLGIRLPL RSGLAGPPLG LRLPRETDEE
SEEPGRGSF VEMVDNLRGK SGQGYYVEMT VGSPPQTLNI LVDTGSSNFA
VGAAPHFPLH RYYQRQLSST YRDLRKGVVV PYTQGKWEGE LGTDLVSIPH
GPNVTVRANI AAITESDKFF INGSNWEGIL GLAYAEIARP DDSLEPFFDS
LVKQTHIPNI FSLQLCGAGF PLNQTEALAS VGGSMIIGGI DHSLYTGS LW
YTPIRREWYY EVIIVRVEIN GQDLKMDCKE YNYDKSIVDS GTTNLR LPKK
VFEAAVKS IK AASSTEKFPD GFWLGEQLVC WQAGTPWNI FPVISLYLMG
EVTNQSFRIT ILPQQYLRPV EDVATSQDDC YKFAVSQSST GTVMGAVIME
GFYVVFDRAR KRIGFAVSAC HVHDEFRTAA VEGPFVTADM EDCGYNIPQT
DESTLMTIAY VMAAICALFM LPLCLMVCQW RCLRCLRHQH DDFADDISLL
K

FIG. 6

MAPALRWLLL WVGSVMLPAQ GTHLGIRLPL RSGLAGPPLG LRLPRETDEE
PEEPGRRGSF VEMVDNLRGK SGQGYVEMT VGSPPQTLNI LVDTGSSNFA
VGAAPHFPLH RYYQRQLSST YRDLRKSVVV PYTQGKWEGE LGTDLVSIPH
GPNVTVRANI AAITESDKFF INGSNWEGIL GLAYAEIARP DDSLEPFFDS
LVKQTHIPNI FSLQLCGAGF PLNQTEALAS VGGSMIIGGI DHSLYTGSLW
YTPIRREWYY EVIIVRVEIN QDQLKMDCKE YNYDKSIVDS GTTNLRLPKK
VFEAAVKSIA AASSTEKFID GFWLGEQLVC WQAGTTPWNI FPVISLYLMG
EVTNQSFRIT ILPQQYLRPV EDVATSQDDC YKFAVSQSST GTVMGAVIME
GFYVVFDRAR KRIGFAVSAC HVHDEFRTAA VEGPFVTADM EDCGYNIPQT
DESTLMTIAY VMAAICALFM LPLCLMVCQW RCLRCLRHQH DDFADDISLL
K

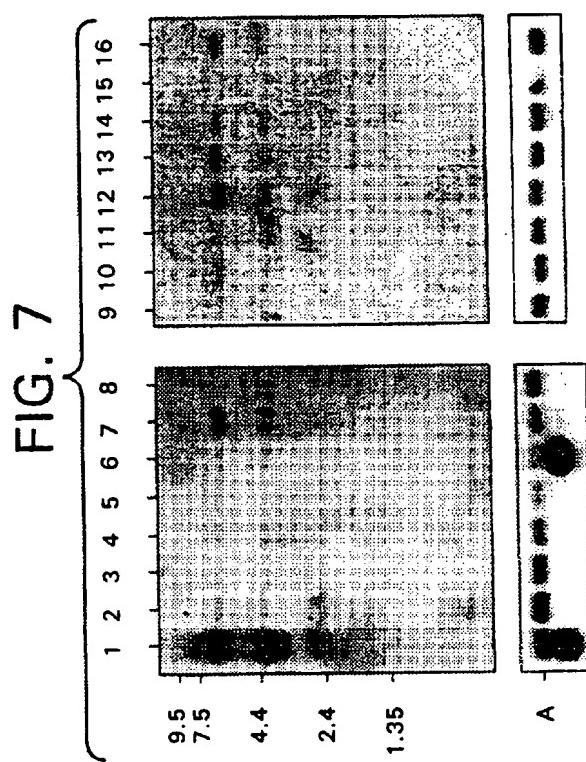


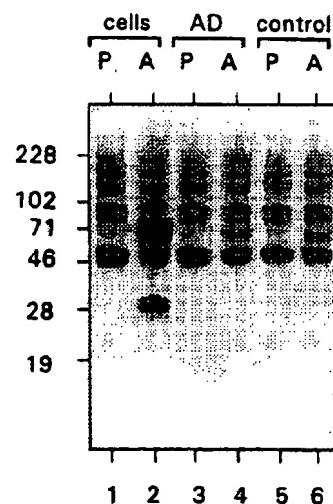
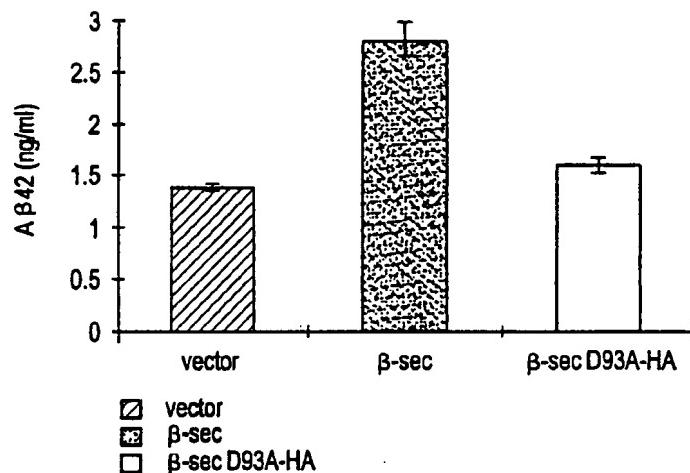
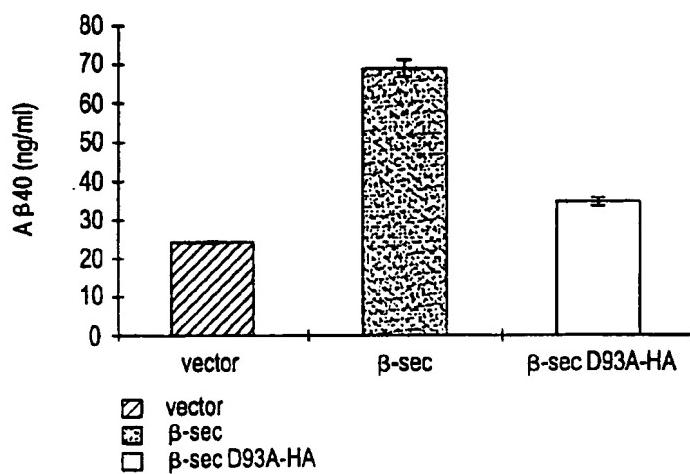
FIG. 8

FIG. 9A**FIG. 9B**

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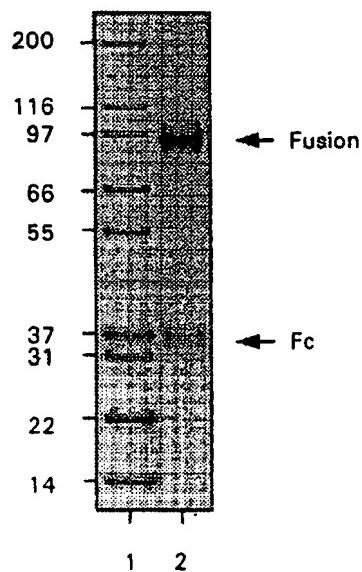
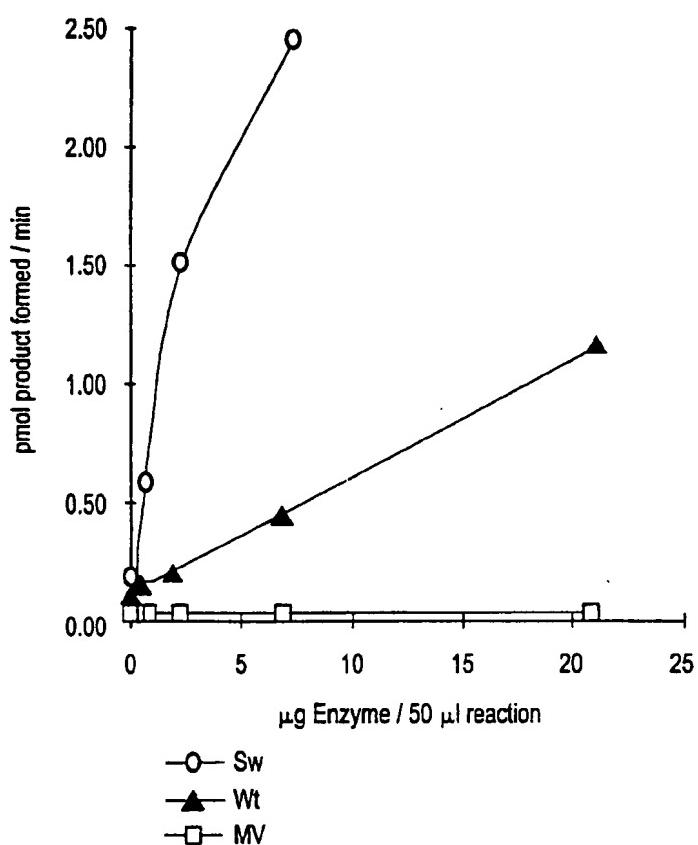
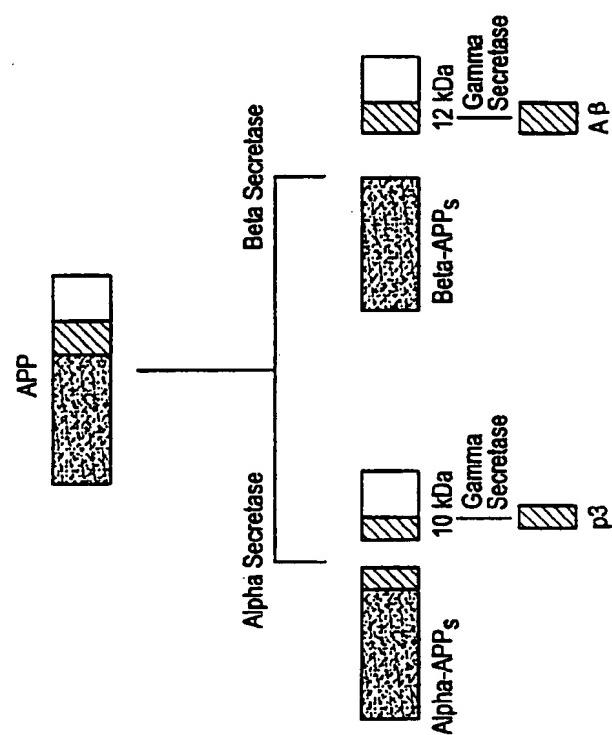
FIG. 10**SUBSTITUTE SHEET (RULE 26)**

FIG. 11



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FIG. 12



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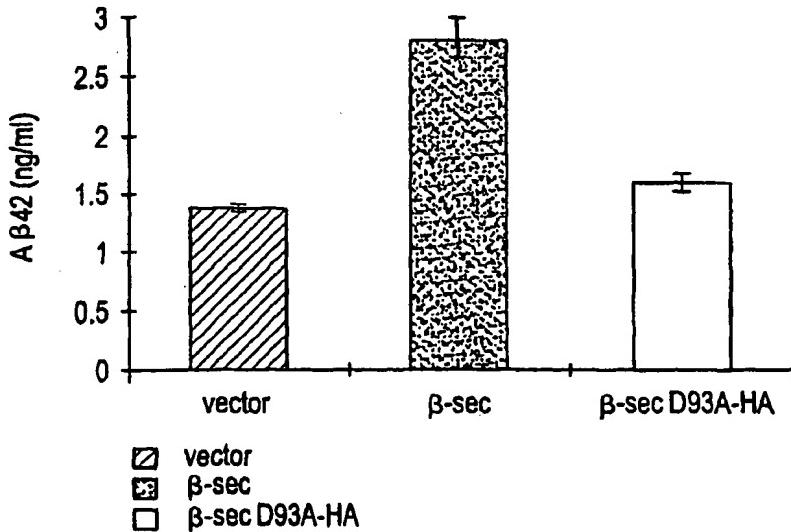
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| (51) International Patent Classification 7 : | A1 | (11) International Publication Number: WO 00/58479 (43) International Publication Date: 5 October 2000 (05.10.00) |
| C12N 15/52, 9/64, C07K 16/40, A61K 38/48, C12N 15/62, A01K 67/027, G01N 33/566, A61K 48/00 | | |
| (21) International Application Number: PCT/US00/07755 | | (81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). |
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| (71) Applicant: AMGEN INC. [US/US]; One Amgen Center Drive, Thousand Oaks, CA 91320-1799 (US). | | |
| (72) Inventors: CITRON, Martin; 1272 Wilder Street, Thousand Oaks, CA 91362 (US). VASSAR, Robert, James; 272 Via Colinas, Westlake Village, CA 91362 (US). BENNETT, Brian, Drake; 2706 Regina Avenue, Thousand Oaks, CA 91360 (US). | | Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |
| (74) Agents: ODRE, Steven, M. et al.; Amgen, Inc., One Amgen Center Drive, Thousand Oaks, CA 91320-1799 (US). | | |

(54) Title: BETA SECRETASE GENES AND POLYPEPTIDES



(57) Abstract

Disclosed are novel genes encoding beta secretase polypeptides. Also disclosed are methods of making and using the polypeptides.

INTERNATIONAL SEARCH REPORT

| |
|-------------------------|
| National Application No |
| PCT/US 00/07755 |

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| A. CLASSIFICATION OF SUBJECT MATTER | | |
| IPC 7 C12N15/52 C12N9/64 C07K16/40 A61K38/48 C12N15/62 A01K67/027 G01N33/566 A61K48/00 | | |
| According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED | | |
| Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12N C07K A61K A01K | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched | | |
| Electronic data base consulted during the international search (name of data base and, where practical, search terms used) STRAND, BIOSIS, EMBASE, MEDLINE, CAB Data, WPI Data, EPO-Internal, PAJ | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| E | WO 00 17369 A (PARODI LUIS A ;BIENKOWSKI MICHAEL JEROME (US); HEINRIKSON ROBERT L) 30 March 2000 (2000-03-30) the whole document ---- | 1-21 |
| P, X | SINHA S ET AL: "PURIFICATION AND CLONING OF AMYLOID PRECURSOR PROTEIN BETA-SECRETASE FROM HUMAN BRAIN" NATURE, GB, MACMILLAN JOURNALS LTD. LONDON, vol. 402, no. 6761, 2 December 1999 (1999-12-02), pages 537-540, XP000881765 ISSN: 0028-0836 the whole document ---- -/-/ | 1-21 |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. | | <input checked="" type="checkbox"/> Patent family members are listed in annex. |
| * Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority (claims) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed | | |
| Date of the actual completion of the international search 10 July 2000 | | Date of mailing of the international search report 24/07/2000 |
| Name and mailing address of the ISA European Patent Office, P.O. 5818 Patenttaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epoN, Fax: (+31-70) 340-3016 | | Authorized officer Hix, R |

INTERNATIONAL SEARCH REPORT

| |
|--|
| National Application No PCT/US 00/07755 |
|--|

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation or document, with indication, where appropriate, of the relevant portions | Relevant to claim No. |
|----------|---|-----------------------|
| P,X | R. YAN ET AL.: "Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity." NATURE, vol. 402, no. 6761, 2 December 1999 (1999-12-02), pages 533-537, XP002141788 the whole document --- | 1-21 |
| P,X | I. HUSSAIN ET AL.: "Identification of a novel aspartic protease (Asp 2) as Beta-secretase." MOLECULAR AND CELLULAR NEUROSCIENCE, vol. 14, no. 6, December 1999 (1999-12), pages 419-427, XP000921315 the whole document | 1-21 |
| P,X | R. VASSAR ET AL.: "Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE." SCIENCE, vol. 286, no. 5440, 22 October 1999 (1999-10-22), pages 735-741, XP000914811 the whole document | 1-21 |
| X | EP 0 855 444 A (SMITHKLINE BEECHAM PLC ;SMITHKLINE BEECHAM CORP (US)) 29 July 1998 (1998-07-29) cited in the application the whole document | 1-21 |
| Y | US 5 744 346 A (KEIM PAMELA S ET AL) 28 April 1998 (1998-04-28) cited in the application the whole document | 1-21 |
| Y | WO 98 26059 A (KEIM PAMELA S ;SINHA SUKANTO (US); ANDERSON JOHN P (US); ATHENA NE) 18 June 1998 (1998-06-18) the whole document | 1-21 |
| P,Y | WO 99 64587 A (BERTRAND PHILIPPE ;MOUTAOUAKIL MOHAMED (FR); MUÑOZ GIMENEZ NOELI () 16 December 1999 (1999-12-16) the whole document | 1-21 |
| | --- | -/- |

INTERNATIONAL SEARCH REPORT

| |
|-------------------------|
| National Application No |
| PCT/US 00/07755 |

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|---|-----------------------|
| A | G. HUBER ET AL.: "cDNA cloning and molecular characterization of human brain metalloprotease MP100: A beta-secretase candidate?" JOURNAL OF NEUROCHEMISTRY, vol. 72, no. 3, March 1999 (1999-03), pages 1215-1223, XP000921316 the whole document --- | |
| A | A. THOMPSON ET AL.: "Expression and characterization of human beta-secretase candidates metalloendopeptidase MP78 and cathepsin D in beta-APP- overexpressing cells" MOLECULAR BRAIN RESEARCH, vol. 48, no. 2, 1997, pages 206-214, XP000923393 the whole document --- | |
| A | N. CHEVALLIER ET AL.: "Cathepsin D displays in vitro beta-secretase-like specificity." BRAIN RESEARCH, vol. 750, 1997, pages 11-19, XP000921314 the whole document --- | |
| A | EP 0 848 062 A (SMITHKLINE BEECHAM PLC ;SMITHKLINE BEECHAM CORP (US)) 17 June 1998 (1998-06-17) cited in the application the whole document ----- | |

INTERNATIONAL SEARCH REPORT

Information on patent family members

| | |
|-------------------------|--|
| National Application No | |
| PCT/US 00/07755 | |

| Patent document cited in search report | | Publication date | Patent family member(s) | | Publication date |
|--|---|------------------|-------------------------|--------------|------------------|
| WO 0017369 | A | 30-03-2000 | AU | 6141899 A | 10-04-2000 |
| EP 0855444 | A | 29-07-1998 | CA | 2221686 A | 28-07-1998 |
| | | | JP | 10327875 A | 15-12-1998 |
| | | | JP | 2000060579 A | 29-02-2000 |
| US 5744346 | A | 28-04-1998 | AU | 6383396 A | 30-12-1996 |
| | | | EP | 0871720 A | 21-10-1998 |
| | | | JP | 11507538 T | 06-07-1999 |
| | | | WO | 9640885 A | 19-12-1996 |
| | | | US | 5942400 A | 24-08-1999 |
| WO 9826059 | A | 18-06-1998 | AU | 1684097 A | 03-07-1998 |
| WO 9964587 | A | 16-12-1999 | FR | 2779444 A | 10-12-1999 |
| | | | AU | 4045599 A | 30-12-1999 |
| EP 0848062 | A | 17-06-1998 | JP | 11069981 A | 16-03-1999 |
| | | | US | 6025180 A | 15-02-2000 |